REMARKS

Claims 5-8 are currently pending. Claims 1-4 and 9-36 are withdrawn from consideration as being drawn to non-elected inventions. Claims 5 and 7 have been amended to better clarify what Applicants believe to be the invention. Support for the amendments can be found throughout the specification, but particularly on page 9, lines 31-32, continuing onto page 10, lines 1-17; and on page 10, lines 27-32, continuing onto page 11, lines 1-19; and on page 12, lines 31-32, continuing onto page 13, lines 1-2; and in Example 2 on pages 73-83. No issue of new matter is believed to be introduced by this amendment. Accordingly, claims 5-8 are currently under consideration.

Rejections under 35 U.S.C. § 101

The Examiner has rejected claims 5-8 under 35 U.S.C. § 101 and alleges that the claimed invention lacks patentable utility. The claims were also rejected as lacking enablement because the Examiner alleges that by not providing a sufficient utility for the claimed inventions, the Applicant has not enabled those in the art to use the claimed invention. These claims broadly read on any peptides comprising a sequence according to the generic formula of SEQ ID NO.: 3, or on peptides comprising SEQ ID NO.: 19.

Furthermore, the Examiner alleges that the Applicant has made no attempt to argue the utility of peptides comprising non-naturally occurring ZA-loop type structures falling within the scope of the formula of SEQ ID NO.: 3. In addition, with respect to the utility rejection over the peptides of SEQ ID NO.: 19, the Examiner alleges that the Applicant traverses this rejection solely on the basis of art teaching the association between bromodomains and HIV infection. However, the art teaches HIV Tat protein association only with the bromodomain of PCAF. There are no teachings either in the art or in the application regarding the association of the ZA loop of SEQ ID NO.: 19 with HIV, or with any other disease, disorder, or pathogen infection. The Examiner further noted that the application itself provides evidence that the HIV Tat protein does not interact with every bromodomain. Furthermore, the present claims are limited to peptides comprising a ZA loop corresponding to SEQ ID NO.: 3. The Examiner alleges that the sequence of P/CAF, as described on Figure 1 of the application, does not fall within the scope of the claimed inventions. Furthermore, the Examiner alleges that because the assertion that the claimed peptides may be found useful to treat unspecified disorders, or to identify compounds that may be so useful, is not considered a sufficiently specific utility to meet 35 U.S.C. 101.

The Examiner alleges that the Applicant has not provided any additional arguments regarding the enablement rejection appurtenant to the utility rejection, and that because the Applicant has not provided any guidance as to what specific use each of the claimed peptides may be applied for, the rejection is maintained.

Applicant respectfully traverses the Examiner's rejection and draws the Examiner's attention to the relevant sections in the specification wherein the utility of the invention is described, in particular the sequences comprising the ZA loop of the bromodomain and its interaction with its acetylated lysine ligand. In particular, on page 8, lines 10-23, Applicants describe that the invention provides support for a method for identifying a potential binding partner for a protein comprising an acetyl lysine, and that one embodiment comprises contacting the protein with the polypeptide comprising a bromodomain. Furthermore, in a preferred embodiment, the bromodomain comprises the amino acid sequence of SEQ ID NO: 3, as well as SEQ ID NO: 19. These sequences may be used in a drug screening assay as a means of determining the interaction between a drug (e.g. an acetyl-lysine analog) and a peptide comprising an acetyl-lysine analog and/or a bromodomain. As noted on page 47, lines 4-8:

> *"Thus, standard high throughput drug screening procedures can be employed using a library of low molecular weight compounds, for example that can be screened to identify a binding partner for the bromodomain. Any such chemical library can be used including those discussed above."*

The Examiner's attention is drawn to the instant application on page 18, lines 12-18, whereby Applicants have identified the key region of the bromodomain for the interaction with its acetyl lysine binding partner:

> *"The present invention further provides a key region of the bromodomain for the interaction with its acetyl-lysine binding partner, the ZA loop. The amino acid sequence of the ZA loop is defined in Figure 1 for a number of bromodomains and is depicted in Figure 2A for P/CAF. In a particular embodiment, the ZA loop has between about 21 and 40 amino acid residues comprising the amino acid sequence:*
>
> $F\ X_{2\text{-}3}\ P\ X_{5\text{-}8}\ J_{P/K/H}\ X\ Y\ J_{Y/F/H}\ X_5\ P\ J_{M/I/V}\ D$ *(SEQ ID NO:3)"*

Furthermore, as noted previously, the present invention also provides the three-dimensional structure of a bromodomain as well as the three-dimensional structure of a

bromodomain-acetyl-histamine complex (the acetyl-histamine being a ligand of the bromodomain). The inventors further note that the structural information provided can be employed in methods of identifying drugs that can modulate the cellular processes that involve bromodomain-acetyl-lysine interactions. More particularly, the Applicants note on page 4, lines 9-13:

"In a particular embodiment, the three-dimensional structural information is used in the identification and/design of an inhibitor of leukemia. In another embodiment, the three-dimensional structural information is used in the identification and/design of an inhibitor of HIV-1 infection and/or AIDS."

The Examiner's attention is further drawn to page 20, lines 27-32, continuing onto page 21, lines 1-16, wherein it states:

"Indeed, ***the bromodomain and lysine-acetylated protein interaction can now be implicated to play a causal role in the development of a number of diseases including cancers such as leukemia.*** *For example, chromatin remodeling plays a central role in the etiology of viral infection and cancer [Archer and Hodin, Curr. Opin. Genet. Biol.* ***9****:171-174 (1999); Jacobson and Pillus, Curr. Opin. Genet. Biol.* ***9****:175-184 (1999)]. Both altered histone acetylation/deacetylation and aberrant forms of chromatin-remodeling complexes are associated with human diseases. Furthermore,* ***chromosomal translocation of various cellular genes with those encoding HATs and subunits of chromatin remodeling complexes have been implicated in leukomogenesis.*** *The MOZ (monocytic leukemia zinc finger) and MLL/ALL-1 genes are frequently fused to the gene encoding the co-activator HAT CBP [Sobulo et al., Proc. Natl. Acad. Sci. USA* ***94****:8732-8737(1997)]. The resulting fusion protein MLL-CBP contains the tandem bromodomain-PHD finger-HAT domain of CBP.* ***It also has been shown that both the bromodomain and HAT domain of CBP are required for leukomogenesis, because deletion of either the bromodomain or the HAT domain results in loss of the MLL-CBP fusion protein's ability for cell transform. These results indicate that the CBP bromodomain, and more particularly, the ZA loop of the CBP bromodomain, is an excellent target for developing drugs that interfere with the bromodomain acetyl-lysine interaction that can be used in the treatment of human acute leukemia.*** *In addition, an antibody (e.g., a humanized antibody) raised specifically against a peptide from the ZA loop of the CBP bromodomain could also be effective for treating these conditions."*

Further support for the utility of the invention can be found on page 21, lines 12-15 as shown below:

"These results indicate that the CBP bromodomain, and more particularly, the ZA loop of the CBP bromodomain, is an excellent target for developing drugs that interfere with the bromodomain acetyl-lysine interaction that can be used in the treatment of human acute leukemia."

Additional support for utility can also be found on page 18, lines 4-10, whereby the inventor describes the relevance of the bromodomain in viral growth and replication, in particular, HIV expression and replication. As noted:

"In addition, as disclosed herein, the gene transactivation of HIV-1 Tat protein requires lysine-acetylation at amino acid residue 50 of Tat (see SEQ ID NO:45) by the transcription co-activator p300/CBP and the subsequent formation of a binding complex between the Tat having the acetylated lysine with P/CAF. The binding complex between P/CAF and Tat is mediated via the bromodomain of P/CAF and the acetylated lysine of Tat. Indeed, this binding is required for the gene transactivation activity of Tat and thus, for HIV-1 expression and replication."

Further support for this utility can be found on page 20, lines 11-32; on page 21, lines 1-30 and on page 73, Example 2.

Applicants further refer the Examiner to the references provided herein as Exhibit A, (Dhalluin et al., Nature, Vol. 399, p. 491-496) and Exhibit B (Mujtaba et al. Molecular Cell (2002), 9:575-588), both which provide support for the role of the ZA loop (such as that described in SEQ ID NO: 3 and SEQ ID NO: 19) of the bromodomain in viral replication, in particular HIV and in tumor cell growth. In particular, Dhalluin et al. describes on page 492, in the first paragraph of the left column, how the ZA loop has a "*defined conformation and is packed against the loop between helices α_B and α_C to form a hydrophobic pocket. These tertiary interactions between the two loops appear to favour the left turn of the ZA loop, resulting in the left turn of the ZA loop, resulting in the left-handed, four helix bundle fold of the bromodomain. The hydrophobic pocket formed by loops ZA and BC is lined by residues Val 752, Ala757, Tyr760, Val 763, Tyr802 and Tyr809 and appears to be a site for protein-protein interaction. Furthermore, the conservation of these residues within the ZA loop, as well as the residues within the α- helical regions, implies that the large family of bromodomains has a similar left-handed four-helix bundle structure.*"

Furthermore, the reference by Mujtaba et al., provided herewith as Exhibit B, on page 576 in the right column, in the paragraph entitled "Structure of the PCAF Bromodomain/Tat Peptide Complex", second paragraph from the end, states in a similar fashion that " *The Tat*

peptide adopts an extended conformation and lies across a pocket formed between the ZA and BC loops (Figure 2B). The side chain of the acetyl-lysine intercalates into the protein hydrophobic cavity and interacts extensively with residues F748, V752, Y760, I764, Y802, and Y809 (Figure 2C)."

More importantly, these references point out the need for identification of small molecules that block the interaction of the bromodomain (such as that in P/CSF) and with its binding partner (such as the acetylated lysine in Tat) to prevent viral replication or to induce apoptosis in tumor cells.

Furthermore, Applicant has amended claims 5 and 7 to recite the utility of the isolated peptides :

".... and wherein said isolated peptide may be used for screening for an inhibitor of the interaction between a bromodomain and an acetylated lysine; and wherein said inhibitor may be used to prevent HIV replication or inhibit tumor cell growth."

Based on the foregoing, withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. § 112

Claims 5-8 are rejected under 35 U.S.C. §112, first paragraph for not fulfilling the enablement requirement. The Examiner alleges that the claims fail to enable the use of the full scope of the claimed peptides. In particular, the Examiner alleges that while the application teaches that the peptides may be used to screen for inhibitors of ZA loop-ligand interactions, the application does not teach the ligands for each of the ZA loop structures that fall within the scope of the claims, nor does the application provide a description of what inhibitors of each of the ZA loop interactions may be used for. The Examiner alleges that while the application provides general suggestions that these inhibitors may be used for the treatment of various diseases, neither the application nor the art associates each of the ZA loops (or the bromodomains from which they are derived) with a specific disease or disorder. In traversal of the rejection, the Applicant asserts that the peptides may be screened for modulators, and that the bromodomain of P/CAF is known to associate with the Tat protein of HIV. Additionally, the Applicant has submitted a declaration of Ming-Ming Zhou (a named inventor of the present application) in support of the enablement of the claimed inventions. However, the Examiner further alleges that the identification of a potential use for a single embodiment where the claim has many potential embodiments, the uses for

which are not known, is insufficient to enable those in the art to make or use the claims to their full scope.

Further, the Examiner alleges that the Declaration previously submitted asserts only two specific uses for which the application is enabled: the inhibition of Tat/PCAF interaction, and the screening for compounds that inhibit ZA loop/ligand interactions. There is no discussion in the Declaration of what other specific disorders may be treated with the claimed peptides or compounds identified through their use in screening methods other than inhibition of Tat/PCAF interaction, and no identification of ligands to bromodomains the ligands to which are not known. As stated above, the Examiner alleges that there is no demonstration in the application that each of the peptides according to SEQ ID NO.:3, or the peptides of SEQ ID NO.:19 in specific, has any association with HIV. Thus, both the application and the Declaration provide a specific potential use for only one of the many embodiments that fall within the scope of the claimed peptides. The Examiner further alleges that there is no guidance as to what other *specific* disorders the other peptides may be useful in treating, or for what disorder the peptides may be useful in identifying therapeutic compounds against.

The Examiner alleges that in order to enable the claimed invention, the Applicant must teach how to use as well as how to make the claimed inventions. In the present case, the Examiner alleges that there is no identification or guidance towards the specific use to which every peptide according to SEQ ID NO.:3, or the specific peptide of SEQ ID NO.:19, may be applied. The application does provide a general use – the identification of compounds that inhibit ZA loop ligand interactions. However, the Examiner alleges that the application does not teach what specific uses these compounds may be applied for. Thus, to practice the claimed invention to its full extent, the Examiner alleges that those in the art would be required to identify, with only little guidance from the application, 1) the ligands for each of the ZA loops falling within the scope of the claims, 2) what, if any, disease or disorder the bromodomain may be associated with, 3) compounds that inhibit the bromodomain/ligand interaction, and 4) determine which, if any, of those compounds may be useful in the treatment of the unidentified disorders. In view of the limited guidance provided by the application with respect to these determinations, and teachings in the art illustrating the breadth of the claims, and limited knowledge regarding the functions and associations of bromodomains, the Examiner alleges that the application has not provided

sufficient information to enable the use of all peptides of SEQ ID NO.:3, or of SEQ ID NO.:19 in particular.

Applicant respectfully traverses the Examiner's rejection and asserts that the support for the role of the bromodomain and its interaction with the acetyl lysine of the Tat protein in HIV can be found on page 21, lines 18-30. More importantly, the application provides evidence that acetylated lysine 50 of Tat specifically binds to the bromodomain of P/CAF. The Examiner's attention is drawn to Figures 5-10 and the results of these experiments, which are shown on page 77. This information, taken together with the fact that Tat is tightly regulated by lysine acetylation, and that HIV-1 Tat transcriptional activity is absolutely required for productive HIV viral replication is supportive of a role for this bromodomain as a drug target for blocking HIV replication in cells.

A ligand for a bromodomain is defined on page 48, lines 22-23, wherein it states:

"A compound is identified as a potential ligand if it binds to the ZA loop of the bromodomain."

As shown on page 51, lines 25-28:

"In a particular embodiment of the present invention the bromodomain-ligand complex is the Tat-P/CAF complex and the compound identified by the screen can used to prevent, retard the progression, treat and/or cure AIDS."

Applicant further asserts that Example 1 on pages 52-62 supports the enablement of the ZA loop of the bromodomain binding to its ligand, which in the matter of the present application is an acetylated lysine, such as that found in acetyl-histamine.

Furthermore, agents that can inhibit the binding of the bromodomain with its binding partner/ligand can be found on page 8, lines 29-32, continuing onto page 9, lines 1-8:

"The present invention further provides agents that can inhibit the binding of a bromodomain with a protein comprising an acetyl-lysine. In one embodiment the agent is ISYGR-AcK-KRRQRR (SEQ ID NO:4). In another embodiment the agent is ARKSTGG-AcK-APRKQL (SEQ ID NO:5). In still another embodiment the agent is QSTSRHK-AcK-LMFKTE (SEQ ID NO:6). In yet another embodiment the agent is an analog of acetyl-lysine (see Figures 12 and 13). One particular analog of acetyl-lysine is acetyl-histamine. In still another embodiment the agent is an antibody that recognizes an acetyl-lysine of a protein binding partner of a bromodomain. In a preferred embodiment the agent is an antibody raised against a ZA loop of a bromodomain. These agents can be used as pharmaceuticals in

compositions that contain a pharmaceutically acceptable carrier for example, or in the various drug assays of the present invention, serving as controls to demonstrate specificity."

Furthermore, Applicants have provided previously a declaration under 37 CFR 1.132 which includes additional support for compounds identified by the methods described herein. The Examiner's attention is drawn to the inventor's declaration whereby compounds have been identified on the basis of the bromodomain and ZA loop sequences and coordinates provided in the instant application. These compounds, while being identified using the information provided in the current application as related to bromodomain structure, are supportive of enablement and further illustrative of the utility of the present invention.

Furthermore, Applicant has amended claims 5 and 7 to recite the utility of the isolated peptides :

".... and wherein said isolated peptide may be used for screening for an inhibitor of the interaction between a bromodomain and an acetylated lysine; and wherein said inhibitor may be used to prevent HIV replication or inhibit tumor cell growth."

Based on the foregoing, withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. § 102

The Examiner's previous rejection of claims 5 and 6 under 35 U.S.C. 102(a) as being anticipated by Dhalluin et al. (Nature 399:491-96) is maintained. While Applicants previously traversed this rejection, since the reference was published by the present inventor less than one year from the priority date of the present application, the Examiner alleges that the reference was "by another", and notes that if in fact the reference is by the Applicants of the present invention, the rejection may be overcome by submission of a declaration under 37 CFR 1.132. Applicants herewith submit a declaration under 37 CFR 1.132 whereby Applicants assert that the reference is not "by another". Withdrawal of this rejection is respectfully requested.

Claims 5 and 6 were rejected under 35 USC 102(b) as being unpatentable over Yang et al. (Nature 382: 319-24). The Examiner alleges that Yang et al. teach the purification of P/CAF, including a fusion protein of P/CAF with a FLAG epitope.

Applicants respectfully traverse the Examiner's rejection and assert that Yang et al. do not teach or suggest that the ZA loop of the P/CAF bromodomain, in particular, the synthetic ZA loop of SEQ ID NO: 3, and the bromodomain sequence of SEQ ID NO: 19, as currently claimed. More particularly, Yang et al do not teach or suggest the use of this

synthetic ZA loop sequence as provided by the present inventors for use in identifying drugs that inhibit the interaction between the bromodomain of P/CAF and the Tat protein such that the inhibitors so identified may be useful for treating HIV infections or tumors. It was only through the teachings of the present application that the particular residues of the ZA loop of the bromodomain were identified as being crucial for the interaction between the bromodomain and the acetylated lysine of the Tat peptide. Furthermore, claims 5 and 7 have been amended to recite "consisting of", rather than "comprising", such that the sequences claimed no longer read on the sequences taught by Yang et al.

The Examiner has rejected claims 5 and 6 under 35 USC 102(b) as being unpatentable in light of Denis and Green (Genes Dev. 10 (3): 261-71). The Examiner alleges that Applicants do not set any limits on what a peptide includes, but indicates that the term does include chimeric or fusion polypeptides and proteins. The Examiner alleges that Denis and Green disclose a protein identified as Ring 3, comprising the sequence of SEQ ID NO: 19. The Examiner further alleges that the reference teaches the recombinant production of the protein as a fusion with a polyhistidine.

Applicants respectfully traverse the Examiner's rejection and assert that claims 5 and 6 of the present invention do not read on SEQ ID NO: 19. Furthermore, Denis and Green do not teach or suggest a ZA loop of a bromodomain that may be used for screening inhibitors of the interaction between a bromodomain , such as that in P/CAF and an acetylated lysine of Tat. Applicants assert that the Denis and Green reference does not teach nor suggest the use of the crucial amino acid residues necessary for the interaction between P/CAF and acetylated lysine of Tat for identifying inhibitors of this interaction, and that such inhibitors could be used to inhibit HIV replication or inhibit tumor cell growth. It was only through the work of the present inventors that such criticality of specific residues for interaction between the ZA loop of the bromodomain and the acetylated lysine of Tat became apparent. Based on the foregoing, withdrawal of the rejection is respectfully requested.

Fees

No fees are believe to be necessitated by the foregoing response. However, if this is in error, authorization is hereby given to charge Deposit Account No. 11-1153 for any underpayment, or credit any overages.

Conclusion

Applicants believe that the foregoing amendments to the claims place the application in condition for allowance. Withdrawal of the rejections is respectfully requested. If a discussion with the undersigned will be of assistance in resolving any remaining issues, the Examiner is invited to telephone the undersigned at (201) 487-5800, ext. 118, to effect a resolution.

Respectfully submitted,

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Attachments: Declaration under 1.132 with Exhibit A (Dhalluin et al., Nature, Vol. 399, p. 491-496) and Exhibit B (Mujtaba et al. Molecular Cell (2002), 9:575-588)

EXHIBIT A

Methods

Plasmids. Portions of HPV-18 E1 were generated as *Nco*I–*Sal*I restriction fragments by PCR with the appropriate synthetic primers, and were cloned into the pTM1 vector, which contains the EMCV (encephalomyocarditis virus) IRES (internal ribosome entry site) and the T7 promoter. Portions of the yeast expression vector for the Ini1/hSNF5 deletion mutants fused to the B42 activation domain were generated by subcloning in-frame PCR fragments of Ini1/hSNF5 into the *Eco*RI–*Xho*I cloning sites of pB42AD. The expression plasmid for HA-Ini1/hSNF5 was constructed so that the HA epitope was fused to the N terminus of Ini1/hSNF5. N-terminal, Flag-tagged E1 was cloned into pFLAG-CMV2 (Eastman Kodak) by PCR. The pCDNA3-Ini1/hSNF5 expression vector was constructed by inserting an *Eco*RI–*Xho*I fragment from the Ini1/hSNF5 open reading frame (generated by PCR with the appropriate synthetic primers) into pCDNA3 (Invitrogen) linearized with *Eco*RI and *Xho*I. The antisense Ini1/hSNF5 expression vector pCDNA3-antisense-Ini1/hSNF5 was constructed by inserting an *Eco*RI–*Hin*dIII fragment from pUC19-Ini1/hSNF5 (generated by ligating the *Eco*RI–Xho1 fragment from pCDNA3-Ini1/hSNF5 with pUC19 linearized with *Eco*RI and *Sal*I) into pCDNA3 linearized with *Hin*dIII and *Eco*RI.

Yeast two-hybrid assay. The yeast strain EGY48 (*MATα his3 trp1 ura3 LexA$_{op(x6)}$-LEU3*) harbouring pLexA-18E1 and pSH18-34 was transformed (by the lithium acetate method) with a HeLa cDNA fusion library (20 μg) cloned into the activation-domain vector pB42AD (Clontech). Transformants were selected by culture on SD/galactose/–Ura/–His/–Trp/–Leu plates for 3 days and were then patched onto fresh SD/glucose/–Ura/–His/–Trp plates. The positive clones were tested for galactose-dependent β-galactosidase activity. β-Galactosidase expression in yeast was assayed as described[24].

Metabolic labelling and immunoprecipitation. Cells were labelled for 4 h using 0.5 mCi of [^{35}S]methionine per 10-cm dish in Dulbecco's modified Eagle's medium (DMEM) containing 5% dialysed fetal bovine serum. For detection of E1–Ini1/hSNF5 immune complexes, about 10^7 cells were used for each reaction. Radiolabelled cells were lysed for 1 h at 4 °C in 1 ml EBC buffer: 50 mM Tris-HCl at pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 200 μM sodium orthovanadate, 1 mM PMSF. The lysate was centrifuged at 14,000*g* for 10 min to pellet debris. After preclearing by incubation for 30 min with protein-G–agarose (25 μl), the supernatant was incubated on a rocker for 1 h with anti-HA or anti-Flag antibody. Precipitates were collected on protein-G–agarose beads, which were washed five times in EBC buffer, resuspended in 50 μl of SDS lysis buffer (20 mM Tris-HCl at pH 7.5, 50 mM NaCl, 0.5% SDS, 1 mM dithiothreitol), and heated to 95 °C for 2 min. Supernatants were removed and reimmunoprecipitated as described; precipitates were analysed by 10% SDS–PAGE and autoradiography.

RT-PCR. Total RNA was extracted from transfected C33A cells using an SV total-RNA isolation system (Promega). cDNA was synthesized from 500 ng of RNA as described[25]. PCR was done with 2 μl cDNA and pairs of oligonucleotide primers designed according to the cDNA sequence, as described[25]; the product was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Transient DNA-replication assay. The transient replication assay has been described[26,27]. Replicated DNA was analysed by agarose-gel electrophoresis and Southern blot hybridization with a random-primed, biotin–dATP-labelled pBluscriptIIKS(–) probe and a chemiluminescent detection method (Phototope-Star detection kit; New England BioLabs).

Mutagenesis. E1 mutants were constructed by using a Quick mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutants were confirmed by DNA sequencing.

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Structure and ligand of a histone acetyltransferase bromodomain

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Histone acetylation is important in chromatin remodelling and gene activation[1–4]. Nearly all known histone-acetyltransferase (HAT)-associated transcriptional co-activators contain bromodomains, which are ~110-amino-acid modules found in many chromatin-associated proteins[5–9]. Despite the wide occurrence of these bromodomains, their three-dimensional structure and binding partners remain unknown. Here we report the solution structure of the bromodomain of the HAT co-activator P/CAF (p300/CBP-associated factor)[10,11]. The structure reveals an unusual left-handed up-and-down four-helix bundle. In addition, we show by a combination of structural and site-directed mutagenesis studies that bromodomains can interact specifically with acetylated lysine, making them the first known protein modules to do so. The nature of the recognition of acetyl-lysine by the P/CAF

bromodomain is similar to that of acetyl-CoA by histone acetyltransferase. Thus, the bromodomain is functionally linked to the HAT activity of co-activators in the regulation of gene transcription.

The P/CAF bromodomain represents an extensive family of bromodomains (Fig. 1). A large number of long-range nuclear Overhauser enhancement (NOE)-derived distance restraints were identified in the nuclear magnetic resonance (NMR) spectra of the P/CAF bromodomain, yielding a well-defined three-dimensional structure (Fig. 2a, b). The structure consists of a four-helix bundle (helices α_Z, α_A, α_B and α_C) with a left-handed twist, and a long intervening loop between helices α_Z and α_A (termed the ZA loop) (Fig. 2c). The four amphipathic α-helices are packed tightly against one another in an antiparallel manner, with crossing angles for adjacent helices of ~16–20°. The up-and-down four-helix bundle can adopt two topological folds with opposite handedness (Fig. 2d). The right-handed four-helix bundle fold is more common and is seen in proteins such as haemerythrin and cytochrome b_{562} (refs 12, 13). The left-handed fold of the bromodomain structure is less common, but is also seen in proteins such as cytochrome b_5 and T4 lysozyme[12,13]. This topological difference arises from the orientation of the loop between the first two helices (Fig. 2d). Right-handed, four-helix-bundle proteins have a relatively short hairpin-like connection between the first two helices, which makes the 'preferred' turn to the right at the top of the first helix[12–14]. In contrast, proteins with the left-handed fold usually have a long loop after the first helix and often contain additional secondary structural elements at the base of the helix bundle[12,13]. In the bromodomain structure, this long ZA loop has a defined conformation and is packed against the loop between helices α_B and α_C (termed the BC loop) to form a hydrophobic pocket. These tertiary interactions between the two loops appear to favour the left turn of the ZA loop, resulting in the left-handed, four-helix-bundle fold of the bromodomain. The hydrophobic pocket formed by loops ZA and BC is lined by residues Val 752, Ala 757, Tyr 760, Val 763, Tyr 802 and Tyr 809 (Fig. 2e), and appears to be a site for protein–protein interactions (see below). The pocket is located at one end of the four-helix bundle, opposite the amino and carboxy termini of the protein. The ZA loop varies in length between different bromodomains, but almost always contains residues corresponding to Phe 748, Pro 751, Pro 758, Tyr 760 and Pro 767 (Fig. 1). The conservation of these residues within the ZA loop, as well as residues within the α-helical regions, implies that the large family of bromodomains has a similar left-handed four-helix bundle structure (Fig. 1).

The modular bromodomain structure supports the idea that bromodomains can act as a functional unit for protein–protein interactions[6]. As bromodomains are found in nearly all known nuclear HATs (A-type) that promote transcription-related acetylation of histones on specific lysine residues, but are not present in cytoplasmic HATs (B-type)[7,8,15,16], we tested whether bromodomains could interact with acetyl-lysine (AcK). We performed NMR titration of the P/CAF bromodomain with a peptide (SGRGKGG–AcK–GLGK) derived from histone H4, in which Lys 8 is acetylated (Lys 8 is the major acetylation site in H4 for GCN5, a yeast homologue of P/CAF[1,17]). We found that the bromodomain did bind the AcK peptide, and that this interaction appeared to be specific, on the basis of the ^{15}N-heteronuclear single quantum coherence (HSQC) spectra which showed that only a limited number of residues underwent chemical shift changes as a function of peptide concentration (Fig. 3a). Conversely, NMR titration of the bromodomain with a non-acetylated but otherwise identical H4 peptide showed no noticeable chemical shift changes, demonstrating that the interaction between the bromodomain and the lysine-acetylated H4 peptide depended on acetylation of lysine. The dissociation constant (K_D) for the AcK peptide was estimated to be 346 ± 54 μM. This binding is probably reinforced through additional interactions between bromodomain-containing proteins and target proteins. Many chromatin-associated proteins contain two or more bromodomains (Fig. 1)[6]. We also observed binding with another lysine-acetylated peptide (RKSTGG–AcK–APRKQ) derived from the main acetylation site on histone H3 (residues 9–20)[17] (data not shown). Our results show that the P/CAF bromodomain can bind AcK peptides in an acetylation-dependent manner.

The bromodomain residues that exhibited the most significant ^{1}H and ^{15}N chemical shift changes on peptide binding are located near the hydrophobic pocket between the ZA and BC loops (Fig. 3b). Because the pattern of amide chemical shift changes was similar for the two different Ack-containing peptides, we surmised that the hydrophobic cavity is the primary binding site for AcK. This hypothesis was supported by the results of titration with acetylhistamine, which mimics the chemical structure of the AcK side chain (Fig. 3c). Both ^{15}N- and ^{13}C-HSQC spectra showed that interaction with acetyl-histamine was also acetylation-dependent, involving the same set of residues that showed chemical shift perturbations with similar concentration dependence (data not shown). The bromodomain did not bind to the amino acids Nε-acetyl-lysine or Nα-acetyl-histidine alone, possibly because of the

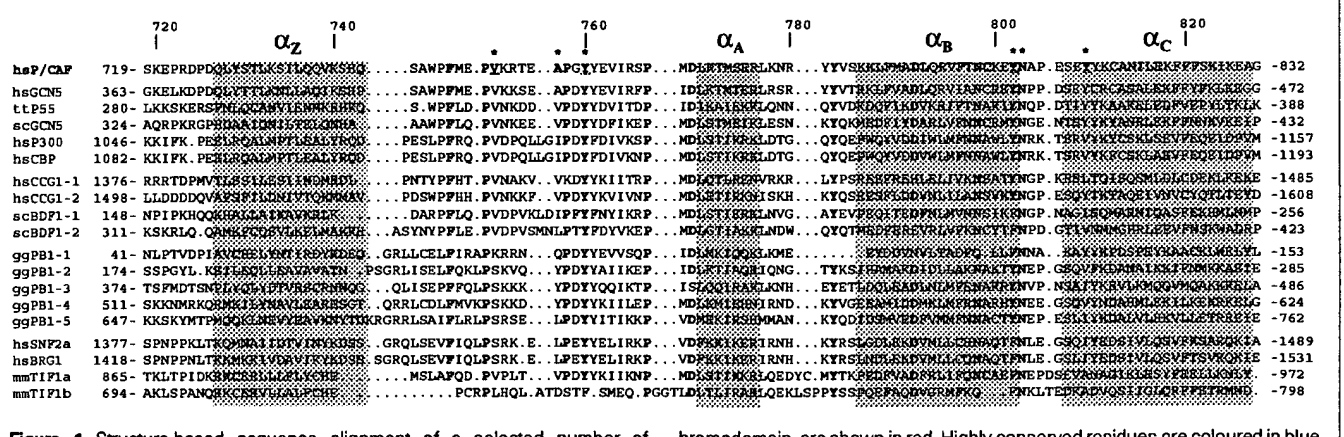


Figure 1 Structure-based sequence alignment of a selected number of bromodomains. The sequences were aligned based on the NMR-derived structure of the P/CAF bromodomain, and the predicated four α-helices are shown in green boxes. Bromodomains are grouped on the basis of the sequence and/or functional similarities as described in ref. 7. Residue numbers of the P/CAF bromodomain are indicated above its sequence. Three absolutely conserved residues, corresponding to Pro 751, Pro 767 and Asn 803 in the P/CAF bromodomain, are shown in red. Highly conserved residues are coloured in blue. Asterisks show the residues of the P/CAF bromodomain that interact with acetylhistamine, as determined by intermolecular NOEs. The underlined residues were changed individually by site-directed mutagenesis to alanine. GenBank accession numbers: *hsP/CAF* U57317, *hsGCN5* U57136, *ttP55* U47321, *scCGN5* Q03330, *hsP300* A54277, *hsCBP* S39162, *hsCCG1* P21675, *scBDF1* P3817, *ggPB1* X90849, *hsSNF2a* S45251, *hsBRG1* S39039, *mmTIF1a* S78219, *mmTIF1b* X99644.

charged amino or carboxylic acid group. Particularly, Nα-acetyl-histidine has a carboxylate group adjacent to the acetyl moiety (Fig. 3c). These results indicate that the P/CAF bromodomain can interact with acetyl-lysine-containing proteins in a specific manner, and that this interaction is localized to the bromodomain hydrophobic cavity.

To identify the key residues involved in bromodomain–AcK recognition, we determined the NMR structure of the P/CAF bromodomain in complex with acetyl-histamine. As anticipated, the acetylated moiety binds in the hydrophobic pocket of the bromodomain (Fig. 4). The intermolecular interactions are mainly hydrophobic, with the methyl group of acetyl-histamine making extensive contacts with the side chains of Val 752, Ala 757 and Tyr 760, and the methylene groups of acetyl-histamine displaying specific NOEs to Val 752, Ala 757, Tyr 760, Tyr 802 and Tyr 809. There were no intermolecular NOEs observed for the imidazole ring

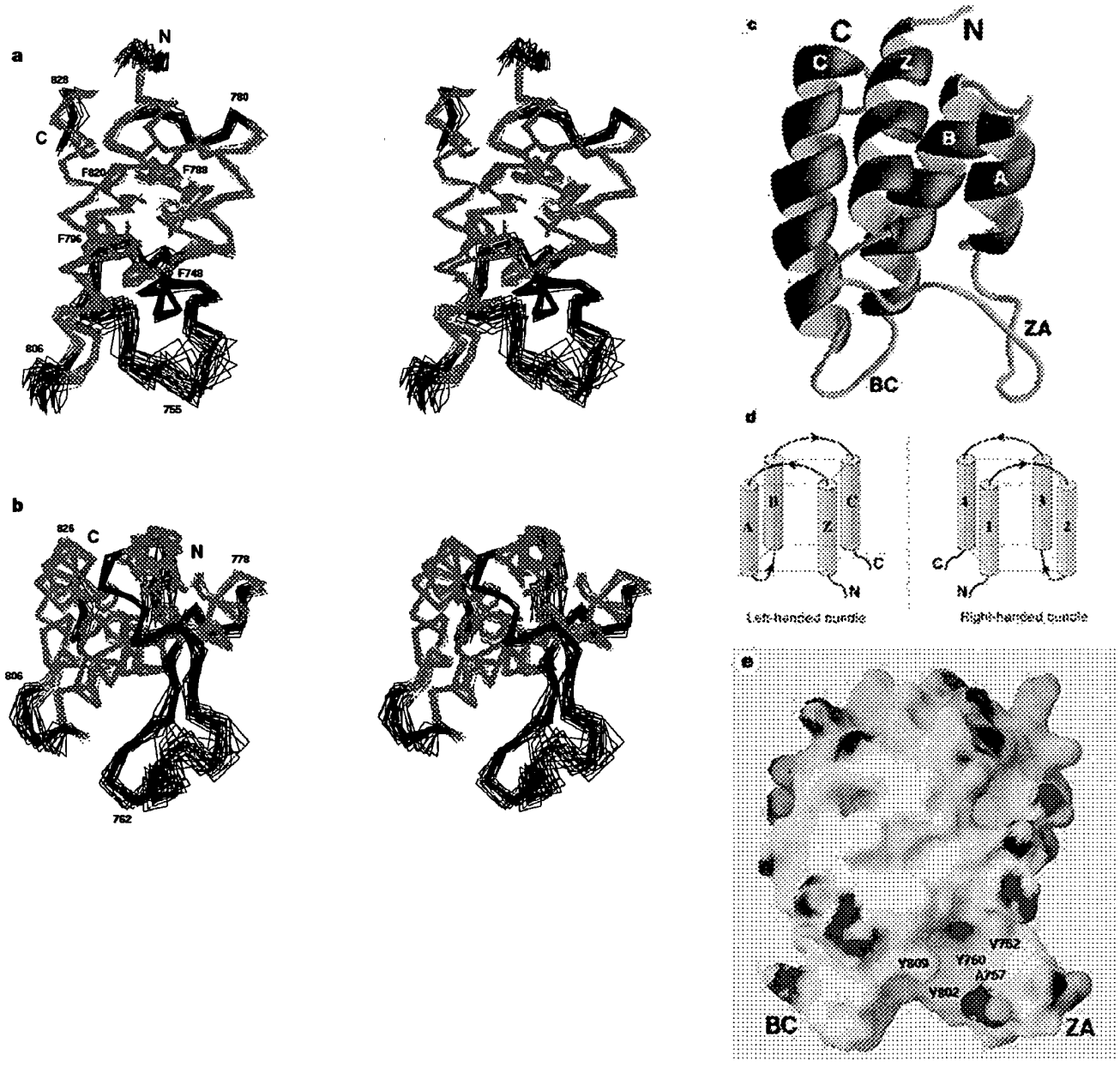
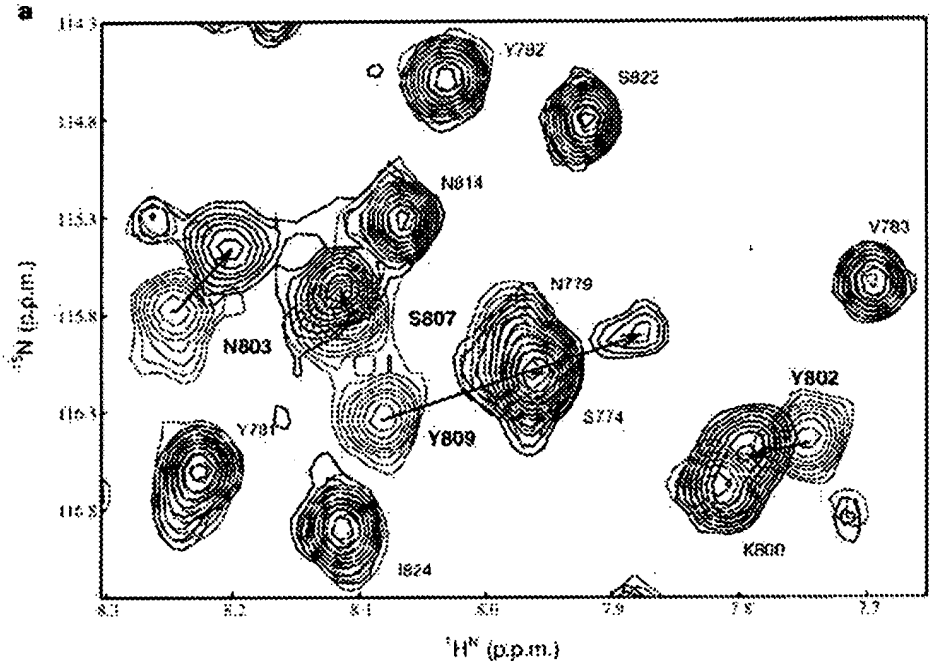
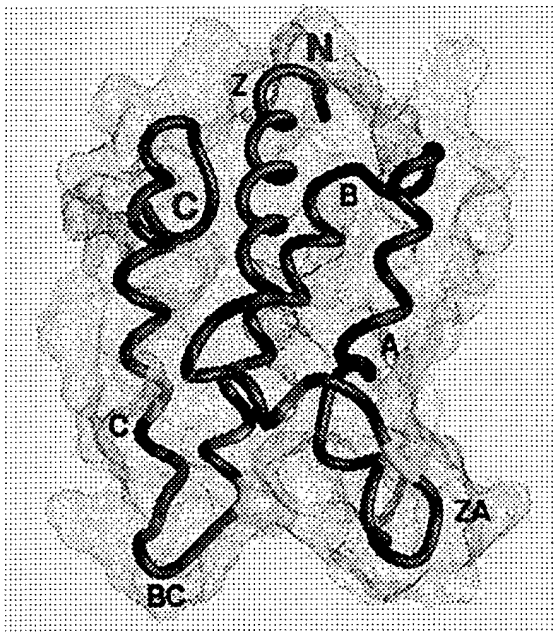


Figure 2 Structure of the P/CAF bromodomain. **a**, Stereoview of the C_α trace of 30 superimposed NMR-derived structures of the bromodomain (residues 722–830). The N-terminal four residues (SKEP) that are structurally disordered are omitted for clarity. For the final 30 structures, the root-mean-square deviations (r.m.s.d.s) of the backbone and all heavy atoms are 0.63 ± 0.11 Å and 1.15 ± 0.12 Å for residues 723–830, respectively. The r.m.s.d.s of the backbone and all heavy atoms for the four α-helices (residues 727–743, 770–776, 785–802 and 807–827) are 0.34 ± 0.04 Å and 0.87 ± 0.06 Å, respectively. **b**, Stereoview of the bromodomain structures from the bottom of the protein, which is rotated ~90° from the orientation in **a**. **c**, Ribbons[29] depiction of the averaged minimized NMR structure of the P/CAF bromodomain. The orientation of **c** is as shown in **a**. **d**, Schematic representation of the overall topology of the up-and-down four-helix bundle folds with opposite handedness. The left-handed fold is seen in bromodomain, cytochrome b_5 and T4 lysozyme (left), whereas the right-handed four-helix bundles are found in proteins such as haemerythrin and cytochrome b_{562} (right)[12,13]. **e**, A molecular surface representation of the electrostatic potential (blue, positive; red, negative) of the bromodomain calculated in GRASP[30]. The hydrophobic and aromatic residues (Tyr 809, Tyr 802, Tyr 760, Ala 757 and Val 752) located between the ZA and BC loops are indicated.

of acetyl-histamine. The spectral analysis shows that the structure of the bromodomain is very similar in both the free and complex forms.

The bromodomain–AcK interaction is similar to that between the histone acetyltransferase Hat1 and acetyl-CoA[18]. Although the binding pockets of these two otherwise structurally unrelated proteins are composed of different secondary structural elements, the nature of acetyl-lysine recognition has striking similarities. In particular, Tyr 809, Tyr 802, Tyr 760 and Val 752 in the bromodomain appear to be related to Phe 220, Phe 261, Val 254 and Ile 217 of Hat1, respectively, in their interactions with the acetyl moiety. This may indicate an evolutionarily convergent mechanism of acetyl-lysine recognition between bromodomains and histone acetyltransferases.





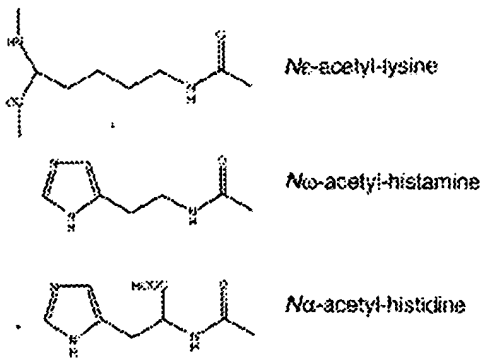


Figure 3 Binding of the P/CAF bromodomain to AcK. **a**, Superimposed region of the 2D ^{15}N-HSQC spectra of the bromodomain (~0.5 mM) in its free form (red) and complexed to the AcK-containing H4 peptide (molar ratio 1:6) (black). **b**, Ribbon and dotted-surface diagram of the bromodomain depicting the location of the lysine-acetylated H4 peptide-binding site. The colour coding reflects the chemical shift changes ($\Delta\delta$) of the backbone amide ^{1}H and ^{15}N resonances upon binding to the AcK peptide as observed in the ^{15}N-HSQC spectra. The normalized weighted average of the chemical shift changes was calculated by $\Delta_{av}/\Delta_{max} = [(\Delta\delta_{NH}^2 + \Delta\delta_N^2/25)/2]^{1/2}/\Delta_{max}$, where Δ_{max} is the maximum weighted chemical shift difference observed for Tyr 809 (0.16 ppm). The backbone atoms are colour-coded in red, yellow or green for residues that have Δ_{av}/Δ_{max} of >0.6 (Tyr 809, Glu 808, Asn 803 and Ala 757), 0.2–0.6 (Ala 813, Tyr 802, Tyr 760 and Val 752) or <0.2 (Cys 812, Ser 807, Cys 799, Phe 796 and Phe 748), respectively. The non-perturbed residues are shown in blue. **c**, Chemical structures of acetyl-lysine, acetyl-histamine, and acetyl-histidine.

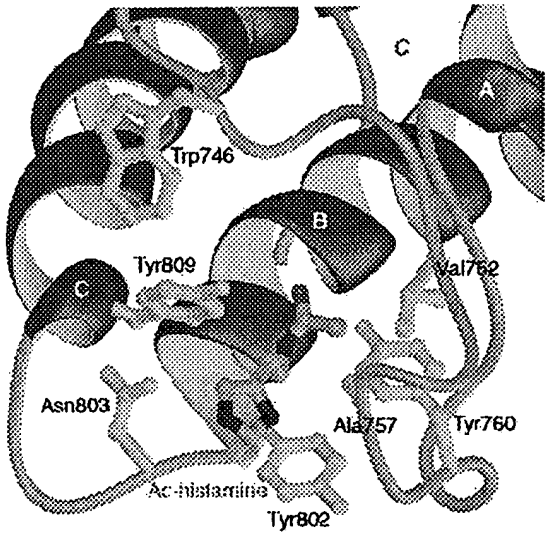


Figure 4 The acetyl-lysine binding pocket. Ribbons[29] depiction of a portion of the P/CAF bromodomain complexed with acetyl-histamine. The ligand is colour-coded by atom type.

To determine the relative contributions of residues within the hydrophobic cavity in bromodomain–AcK binding, we used site-directed mutagenesis to alter residues Tyr 809, Tyr 802, Tyr 760 and Val 752 (Table 1). Substitution of alanine for Tyr 809 completely abrogated the bromodomain binding to the lysine-acetylated H4 peptide, whereas the Y802A, Y760A and V752A mutants had significantly reduced ligand-binding affinity. To test whether these mutations disrupted the overall bromodomain fold, we compared the ^{15}N-HSQC spectra of the mutants to that of the wild-type protein. For the Y809A mutant, the amide chemical shifts were only affected for a few residues near the mutation site. However, mutations of the other residues in the hydrophobic binding pocket caused greater changes in the local protein conformation, particularly in the ZA loop (Table 1). Thus, the results of NMR structural analysis and mutagenesis show that Tyr 809, which is structurally supported by Trp 746 and Asn 803 (Fig. 4), is essential for the bromodomain interaction with the acetyl group of acetyl-lysine, and that Tyr 802, Tyr 760 and Val 752 probably have both structural and functional roles in the recognition. These residues are highly conserved throughout the bromodomain family (Fig. 1), indicating that recognition of acetyl-lysine may be a general feature of bromodomains.

Table 1 Structural and functional analysis of the P/CAF bromodomain mutants

Bromodomain proteins	Structural integrity*	H4 AcK-peptide binding K_D (μM)†
Wild type	++++	346 ± 54
Y809A	++++	No binding‡
Y802A	+++	>10,000§
Y760A	+++	>10,000
V752A	++	>10,000

* We assessed the effects of mutations on the structural integrity of the bromodomain using the ^{15}N-HSQC spectra. The amide ^{1}H/^{15}N resonances of the mutant proteins were compared to those of the wild-type bromodomain to determine whether the particular mutations led to global or local structure disruption. Severe line-broadening of the amide resonances indicates protein conformational exchange due to decreased structural stability resulting from point mutations. Structural integrity of the mutant proteins is expressed here relative to that of the wild type: ++++, as stable as wild type; +++, mildly destabilized, ++, moderately destabilized; –, completely unfolded.
† The ligand-binding affinity (K_D) of the bromodomain proteins was estimated by the following chemical shift changes of amide peaks in the ^{15}N-HSQC spectra as a function of the ligand concentration.
‡ There was no detectable ligand binding in the NMR titration.
§ Ligand-binding affinity was significantly reduced and beyond the limit for reliable measurements by NMR titration.

In conclusion, our results indicate that the bromodomains are acetyl-lysine-binding domains, which, to our knowledge, makes them the first protein modules to exhibit such interactions. Like other modular domains, such as Src homology-2 (SH2) and phosphotyrosine-binding (PTB) domains[19], that specifically interact with phosphotyrosine-containing proteins, the bromodomain/acetyl-lysine recognition could regulate protein–protein interactions by lysine acetylation. Our finding may explain why the bromodomain is indispensable for the function of GCN5 in yeast[20], and supports the hypothesis that bromodomains contribute to highly specific histone acetylation by tethering transcriptional HATs to specific chromosomal sites[1]. Bromodomain–AcK binding could also be important for the assembly and activity of multi-protein complexes in transcriptional activation. Our results should help in the determination of specific molecular mechanisms used by the family of bromodomains in chromatin remodelling and transcriptional activation. □

Methods

Sample preparation. The bromodomain of P/CAF (residues 719–832) was subcloned into the pET14b expression vector (Novagen) and expressed in *Escherichia coli* BL21(DE3) cells. We prepared uniformly ^{15}N- and ^{15}N/^{13}C-labelled proteins by growing bacteria in a minimal medium containing $^{15}NH_4Cl$ with or without $^{13}C_6$-glucose. A uniformly ^{15}N/^{13}C-labelled and fractionally deuterated protein sample was prepared by growing the cells in 75% $^{2}H_2O$. The bromodomain was purified by affinity chromatography on a nickel–IDA column (Invitrogen), followed by the removal of the poly-His tag by thrombin cleavage. The final purification of the protein was achieved by size-exclusion chromatography. We prepared acetyl-lysine-containing peptides on a MiliGen 9050 peptide synthesiser (Perkin Elmer) using Fmoc/HBTU chemistry. Acetyl-lysine was incorporated using the reagent Fmoc-Ac-Lys with HBTU/DIPEA activation. NMR samples contained ~1 mM protein in 100 mM phosphate buffer, pH 6.5, and 5 mM perdeuterated DTT and 0.5 mM EDTA in $H_2O/^{2}H_2O$ (9/1) or $^{2}H_2O$.

NMR spectroscopy. We acquired NMR spectra at 30 °C on a Bruker DRX600 or DRX500 spectrometer. The backbone ^{1}H, ^{13}C and ^{15}N resonances were assigned by using deuterium-decoupled triple-resonance experiments of HNCACB and HN(CO)CACB[21], recorded with the uniformly ^{15}N/^{13}C-labelled and fractionally deuterated protein. The side-chain atoms were assigned from 3D HCCH-TOCSY[22] and (H)C(CO)NH-TOCSY[23] data collected on the uniformly ^{15}N/^{13}C-labelled protein. Stereospecific assignments of the methyl groups of valine and leucine residues were obtained using a fractionally ^{13}C-labelled sample[24]. The NOE-derived distance restraints were obtained from ^{15}N- or ^{13}C-edited 3D NOESY spectra[22]. We determined ϕ-angle restraints based on the $^{3}J_{HN,H\alpha}$ coupling constants measured in a 3D HNHA spectrum[22]. Slowly exchanging amide protons were identified from a series of 2D ^{15}N-HSQC spectra recorded after the H_2O buffer was changed to a $^{2}H_2O$ buffer. The intermolecular NOEs used in defining the structure of the bromodomain/acetyl-histamine complex were detected in ^{13}C-edited (F_1), ^{13}C/^{15}N-filtered (F_3) 3D NOESY spectra[22]. All NMR spectra were processed with the NMRPipe/NMRDraw programs and analysed using NMRView[25].

Structure calculations. Structures of the bromodomain were calculated with a distance geometry/simulated annealing protocol using the X-PLOR program[26]. A total of 1,324 manually assigned NOE-derived distance restraints were obtained from the ^{15}N- and ^{13}C-edited NOE spectra. We further analysed the NOE spectra by the iterative automated assignment procedure using ARIA[27], which integrates with X-PLOR for structure calculations. A total of 1,519 unambiguous and 590 ambiguous distance restraints were identified from the NOE data by ARIA, many of which were checked and confirmed manually. The ARIA-assigned distance restraints agreed with the structures calculated by using only the manually assigned NOE distance restraints, 28 hydrogen-bond distance restraints for 14 hydrogen bonds, and 54 ϕ-angle restraints. The final structure calculations employed a total of 3,515 NMR experimental restraints obtained from the manual and the ARIA-assisted assignments, 2,843 of which were unambiguously assigned NOE-derived distance restraints (1,077 intra-residue, 621 sequential, 550 medium-range and 595 long-range NOEs). For the ensemble of the final 30 structures, no distance and torsional angle restraints

were violated by more than 0.3 Å and 5°, respectively. The total distance violation, and dihedral violation energies were 178.7 ± 2.4, 41.6 ± 0.9 and 0.50 ± 0.06 kcal mol^{-1}, respectively. The Lennard-Jones potential, which was not used during any refinement stage, was −526.2 ± 16.8 kcal mol^{-1} for the final structures. Ramachandran plot analysis of the final structures (residues 727–828) with Procheck-NMR[28] showed that 71.0 ± 0.6%, 23.8 ± 0.6%, 3.5 ± 0.2% and 1.7 ± 0.2% of the non-glycine and non-proline residues were in the most favourable, additionally allowed, generously allowed and disallowed regions, respectively. The corresponding values for the residues in the four α-helices (residues 727–743, 770–776, 785–802 and 807–827) were 88.9 ± 0.4%, 11.0 ± 0.4%, 0.1 ± 0.1% and 0.0 ± 0.0%, respectively. The structure of the bromodomain/acetyl-histamine complex was determined using the free-form structure and an additional 25 intermolecular and 5 intra-ligand NOE-derived distance restraints.

Site-direction mutagenesis. We prepared mutant proteins using the QuikChange site-directed mutagenesis kit (Stratagene). The presence of appropriate mutations was confirmed by DNA sequencing.

Ligand titration. We performed ligand titration by recording a series of 2D ^{15}N- and ^{13}C-HSQC spectra on the uniformly ^{15}N- and ^{15}N/^{13}C-labelled bromodomain (~0.3 mM), respectively, in the presence of different amounts of ligand concentration ranging from 0 to ~2.0 mM. The protein sample and the stock solutions of the ligands were all prepared in the same aqueous buffer containing 100 mM phosphate and 5 mM perdeuterated DTT at pH 6.5.

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Correspondence and requests for materials should be addressed to M.M.Z. (e-mail: zhoum@inka.mssm.edu). Coordinates for the NMR structure of the P/CAF bromodomain have been deposited in the Brookhaven Protein Data Bank under accession code 1B91.

Enzyme dynamics and hydrogen tunnelling in a thermophilic alcohol dehydrogenase

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Biological catalysts (enzymes) speed up reactions by many orders of magnitude using fundamental physical processes to increase chemical reactivity. Hydrogen tunnelling has increasingly been found to contribute to enzyme reactions at room temperature[1]. Tunnelling is the phenomenon by which a particle transfers through a reaction barrier as a result of its wave-like property[1–3]. In reactions involving small molecules, the relative importance of tunnelling increases as the temperature is reduced[4]. We have now investigated whether hydrogen tunnelling occurs at elevated temperatures in a biological system that functions physiologically under such conditions. Using a thermophilic alcohol dehydrogenase (ADH), we find that hydrogen tunnelling makes a significant contribution at 65 °C; this is analogous to previous findings with mesophilic ADH at 25 °C (ref. 5). Contrary to predictions for tunnelling through a rigid barrier, the tunnelling with the thermophilic ADH decreases at and below room temperature. These findings provide experimental evidence for a role of thermally excited enzyme fluctuations in modulating enzyme-catalysed bond cleavage.

Structural studies of enzymes have influenced our view of catalysis enormously by providing three-dimensional pictures of active sites. But although these time-averaged structures are extremely useful as a starting point for evaluating and modelling catalysis, they generally fail to incorporate the wide range of dynamic motion that can occur in proteins[6]. It is a major experimental challenge to examine the relationship between the dynamics of a protein and its activity. Various studies have shown a relationship between protein dynamics and the physical steps of the enzymatic process, that is, those steps involving the formation and breakdown of catalytic complexes[7,8], but a far greater problem is whether protein dynamics have a direct role in the catalysis of bond forming and cleaving. Our present findings on hydrogen transfer under physiological conditions cannot be explained without invoking both quantum mechanics and enzyme dynamics.

Recent literature has demonstrated the importance of quantum-mechanical tunnelling in enzyme-catalysed hydrogen-transfer reactions[1]. Indirect evidence that dynamical motion is involved comes from the demonstration that surface glycosylation of glucose oxidase decreases the extent of quantum behaviour observed in

EXHIBIT B

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Structural Basis of Lysine-Acetylated HIV-1 Tat Recognition by PCAF Bromodomain

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Summary

The human immunodeficiency virus type 1 (HIV-1) *trans*-activator protein Tat stimulates transcription of the integrated HIV-1 genome and promotes viral replication in infected cells. Tat transactivation activity is dependent on lysine acetylation and its association with nuclear histone acetyltransferases p300/CBP (CREB binding protein) and p300/CBP-associated factor (PCAF). Here, we show that the bromodomain of PCAF binds specifically to HIV-1 Tat acetylated at lysine 50 and that this interaction competes effectively against HIV-1 TAR RNA binding to the lysine-acetylated Tat. The three-dimensional solution structure of the PCAF bromodomain in complex with a lysine 50-acetylated Tat peptide together with biochemical analyses provides the structural basis for the specificity of this molecular recognition and reveals insights into the differences in ligand selectivity of bromodomains.

Introduction

The human immunodeficiency virus type 1 (HIV-1) protein Tat is an atypical *trans*-activator of transcription which functions through binding to an RNA element known as the transactivation responsive region (TAR), located in the retroviral long-terminal repeat (LTR) (Cullen, 1998; Jeang et al., 1999; Kam, 1999). Tat binds to TAR RNA with high affinity but transiently (Keen et al., 1997; Rana and Jeang, 1999). Dissociation of Tat from TAR RNA facilitates Tat association with the assembled RNA polymerase II (RNAPII) complex (Deng et al., 2000; Kiernan et al., 1999). The latter process enables the transcriptional machinery complex to elongate efficiently on the viral DNA template in order to produce full-length HIV transcripts during viral productive replication in infected cells (Adams et al., 1994; Garber and Jones, 1999).

While the detailed molecular mechanisms underlying Tat dissociation from TAR RNA and its transactivation of transcription of the integrated HIV-1 genome remain elusive, increasing lines of evidence suggest that Tat activity requires its association with several multiprotein complexes, which include the cyclinT1/cyclin-dependent kinase 9 (CDK9) complex (Jones, 1997; Wei et al., 1998) and histone acetyltransferase (HAT) transcriptional coactivators, p300/CBP (CREB binding protein), and p300/CBP-associated factor (PCAF) (Benkirane et al., 1998; Deng et al., 2000; Hottiger and Nabel, 1998). Recruitment of CDK9 through the N-terminal cysteine-rich region of Tat results in hyperphosphorylation of the C-terminal domain of RNAPII that increases elongation efficiency of HIV-1 transcription (Wei et al., 1998). Recently, it has been shown that Tat activity is dependent on acetylation by p300/CBP at K50 located in the C-terminal arginine-rich motif (ARM) (Kiernan et al., 1999; Ott et al., 1999), a region that is also important for TAR RNA binding and nuclear localization. Mutation of K50 to arginine, a conserved amino acid substitution that retains the positive charge but prevents acetylation by p300, markedly decreases the synergistic activation of the HIV-1 promoter by Tat and p300 (Kiernan et al., 1999; Ott et al., 1999). Tat acetylation at K50 results in its dissociation from TAR RNA and promotes formation of a multiprotein complex comprised of Tat, p300/CBP, and PCAF (Benkirane et al., 1998; Deng et al., 2000). Furthermore, it has been shown that the HAT activity of PCAF is preferentially required for Tat transactivation of transcription of the integrated but not the unintegrated HIV-1 LTRs (Benkirane et al., 1998).

Protein lysine acetylation is emerging as a central mechanism in regulation of chromatin remodeling and transcriptional activation (Jenuwein and Allis, 2001; Kouzarides, 2000; Strahl and Allis, 2000). Bromodomains, an extensive family of conserved protein modules found in many chromatin-associated proteins and in nearly all nuclear histone acetyltransferases (Brownell and Allis, 1996; Haynes et al., 1992; Jeanmougin et al., 1997; Tamkun et al., 1992), have been recently discovered to function as acetyl-lysine binding domains (Dhalluin et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Owen et al., 2000). This new finding suggests a novel mechanism for regulating protein-protein interactions via lysine acetylation (Dyson et al., 2001; Jenuwein and Allis, 2001; Strahl and Allis, 2000; Winston and Allis, 1999). This new mechanism supports the hypothesis that bromodomains could contribute to highly specific histone acetylation by tethering transcriptional HATs to specific chromosomal sites (Brownell and Allis, 1996; Manning et al., 2001; Travers, 1999), and to the assembly and activity of multiprotein complexes of chromatin remodeling such as SAGA and NuA4 (Brown et al., 2001; Sterner et al., 1999). However, because no specific, biologically relevant binding sites had been reported for any particular bromodomain, the major question of ligand specificity of bromodomains still remains unanswered.

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In efforts to determine the mechanisms of action of Tat in transactivation of HIV-1 transcription, we investigated whether the interaction of the activated, lysine-acetylated Tat with the nuclear HAT transcriptional cofactors p300/CBP and PCAF involves any of the bromodomains of the latter proteins. Here, we report that the bromodomain of PCAF but not CBP can specifically recognize the lysine-acetylated Tat at K50 (not K51 or K28), and this interaction competes effectively against TAR RNA binding to the acetylated Tat. We have also determined the three-dimensional structure of the PCAF bromodomain in complex with a lysine-acetylated peptide derived from Tat at K50 by using nuclear magnetic resonance (NMR) spectroscopy. NMR structural and biochemical analyses were further used to gain structural insights into this important molecular recognition as well as the differences in ligand selectivity of different bromodomains.

Results and Discussion

PCAF Bromodomain Recognition of Lysine-Acetylated HIV-1 Tat at K50

To test whether Tat-p300/CBP-PCAF association involves bromodomains of the histone acetyltransferase transcriptional coactivators, we performed an in vitro binding assay by using recombinant and purified GST-fusion bromodomains and lysine-acetylated peptides derived from known acetylation sites in Tat at K28 and K50. A lysine-acetylated Tat peptide containing AcK50 (SYGR-AcK-KRRQR, where AcK is an *N*$^{\epsilon}$-acetyl-lysine) showed no detectable interactions with either bromodomains or bromodomain and PHD finger (Aasland et al., 1995) tandem modules from CBP or TIF1β (transcriptional intermediary factor 1β, also named KAP-1 and KRIP-1) (Friedman et al., 1996) (Figure 1A). Strikingly, the same Tat peptide bound tightly to the bromodomain of PCAF, which shares high sequence homology to CBP bromodomain (Jeanmougin et al., 1997). The binding is dependent on acetylation of K50 on Tat. Neither of these bromodomains interacted with an acetylated Tat peptide derived from K28 (TNCYCK-AcK-CCFH) (data not shown), highlighting the selective nature of PCAF bromodomain recognition of the Tat AcK50 sequence.

We performed an NMR study in order to determine the specificity of PCAF bromodomain binding to lysine-acetylated Tat. As anticipated, PCAF bromodomain did not bind to Tat AcK28 peptide, nor did CBP bromodomain to either Tat AcK28 or AcK50 peptide. In contrast, PCAF bromodomain bound to Tat AcK50 peptide with high affinity and caused extensive chemical shift perturbations of protein amide resonances, significantly greater than those seen with an acetylated peptide derived from histone H4 at K16, as shown in 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra (Figure 1B). This observation agrees with the differences in dissociation constants (K_D), determined by NMR titration to be ~10 μM and >300 μM for the former and latter complexes, respectively. These results argue that PCAF bromodomain binding to H4 peptide is largely limited to the acetyl-lysine, whereas its recognition of Tat most likely involves additional interactions with residues flanking AcK50.

To assess the biological relevance of the PCAF bromodomain and Tat interaction to the activation of Tat transcriptional activity by PCAF and p300/CBP (Benkirane et al., 1998; Kiernan et al., 1999), we performed cell transfection experiments and measured their combined effect on the activity of the HIV-1 promoter using an HIV-1 LTR-luciferase reporter gene assay (Bieniasz et al., 1998; Madore and Cullen, 1993). As shown in Figure 1C, synergistic activation of Tat-mediated transcription of the HIV-1 promoter in human 293T cells is dependent upon both PCAF and CBP. The latter HAT transcriptional coactivator has been recently shown to be responsible for lysine acetylation of Tat at K50 that is required for Tat activation (Kiernan et al., 1999; Ott et al., 1999). More importantly, our data show that cotransfection of the PCAF bromodomain but not the CBP bromodomain resulted in a significant reduction of the synergistic activation of Tat by PCAF and CBP, likely due to an effective competition of the PCAF bromodomain against the full-length PCAF binding to Tat. Collectively, our in vivo transfection study further confirms the highly specific nature of PCAF bromodomain/Tat recognition and highlights the functional importance of this bromodomain interaction in the synergistic PCAF- and CBP-induced activation of Tat transcriptional activity in HIV-1 gene expression.

Structure of the PCAF Bromodomain/Tat Peptide Complex

To understand the structural basis of this molecular recognition, we determined the three-dimensional structure of the PCAF bromodomain in complex with Tat AcK50 peptide from a total of 2903 NMR-derived restraints. The structure for the protein (residues 723–830) and the peptide (residues 47–54) complex was well defined by the NMR data (Figure 2A, Table 1). The structure of the bromodomain when complexed to the Tat peptide consists of a left-handed four-helix bundle (helices α_Z, α_A, α_B, and α_C) and is similar to its free form structure (Dhalluin et al., 1999), except for the ZA and BC loops that comprise the acetyl-lysine binding site and undergo local conformational changes to accommodate peptide binding (see below). The Tat peptide adopts an extended conformation and lies across a pocket formed between the ZA and BC loops (Figure 2B). The side chain of the acetyl-lysine intercalates into the protein hydrophobic cavity and interacts extensively with residues F748, V752, Y760, I764, Y802, and Y809 (Figure 2C). Peptide residues flanking the acetyl-lysine contact the protein. Particularly, G(AcK−2), R(AcK−1), and R(AcK+3) showed intermolecular NOEs to the protein, and extensive interactions were observed between the side chains of Y(AcK−3) and V763 and between Q(AcK+4) and E756. These specific interactions confer a highly selective association between PCAF bromodomain and Tat.

Structural comparison of PCAF bromomdomain in the free and ligand-bound forms reveals that structural changes of the protein, largely localized in the ZA and BC loops, are directly coupled with the peptide binding (Figure 2D). These structural changes are supported by extensive NMR data, which include changes of chemical shifts and NOE patterns for the backbone amides, side chain methyl groups, and aromatic rings of many protein

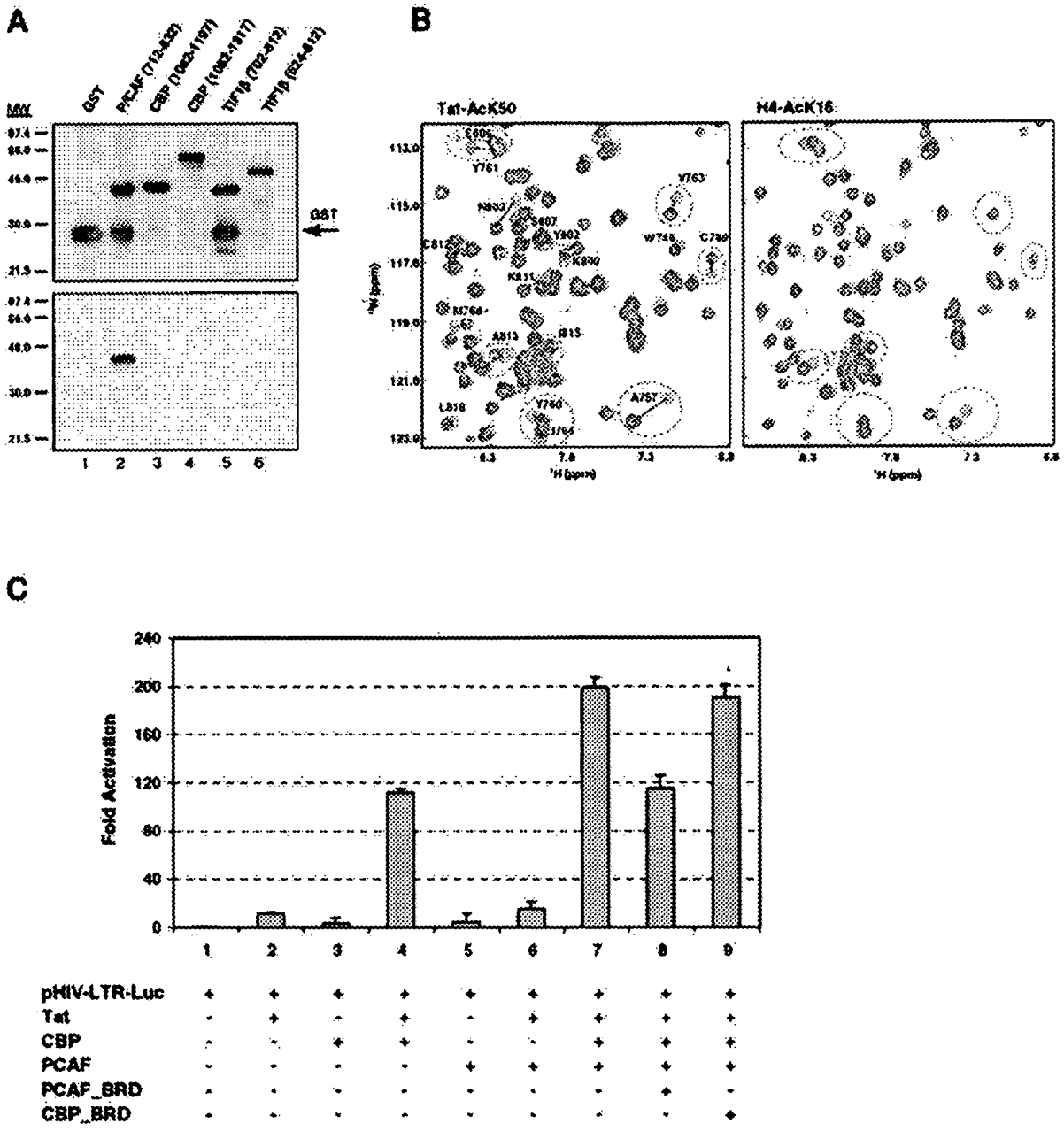


Figure 1. Recognition of Lysine-Acetylated HIV-1 Tat by PCAF Bromodomain

(A) Binding of bromodomains alone from PCAF, CBP, and TIF1β or in combination with PHD fingers from CBP and TIF1β to a biotinylated and lysine-acetylated Tat AcK50 peptide immobilized on streptavidin agarose beads. The upper panel shows purity of the GST-fusion bromodomains used in the assay, separated by SDS-PAGE and stained with Coomassie blue. The lower panel depicts Western blot with anti-GST antibodies, showing specific interaction between PCAF bromodomain and Tat AcK50 peptide.

(B) Comparison of PCAF bromodomain binding to lysine-acetylated peptide derived from HIV-1 Tat at K50 (SYGR-AcK-KRRQR) (left) versus one derived from histone H4 at K16 (SGRGKGGKGLGKGGA-AcK-RHRK) (right). The protein samples were completely titrated with the lysine-acetylated peptide of Tat or H4 with molar ratio 1:1.5 or 1:6, respectively. The 2D ^{1}H-^{15}N HSQC spectra of the bromodomain show changes of backbone amide resonances of the protein in the presence (red) or absence (black) of the peptide ligand. Blue dashed cycles highlight protein residues that exhibited major chemical shift perturbations upon Tat AcK50 peptide binding (left), significantly greater than those observed upon addition of the histone H4 peptide (right).

(C) Functional contribution of PCAF bromodomain and Tat interaction to synergistic stimulation of Tat transcriptional activity by PCAF and CBP. The plasmids used in various combinations in transfections with human 293T cells are as indicated below the graph. The amounts of the plasmids used in transfection experiments are pHIV-LTR-Luc (100 ng), pcTat (100 ng), pRSV-HA-CBP (2.0 ug), pCI-FLAG-PCAF (2.0 ug), pCMV-HA-PCAF_BRD (0.5 ug), and pCMV-FLAG-CBP_BRD (0.5 ug). Total amounts of DNA for transfections were kept constant with addition of empty control vector. Luciferase activities of the cell cytoplasmic extracts were measured using a luciferase-based assay (Promega) 24 hr after transfection and normalized to the β-galactosidase plasmid uptake as described in the Experimental Procedures. Fold activation in 293T cells is expressed relative to the basal expression of pHIV-LTR-Luc set as 1. Mean values of the luciferase activities represent at least three independent transfection experiments.

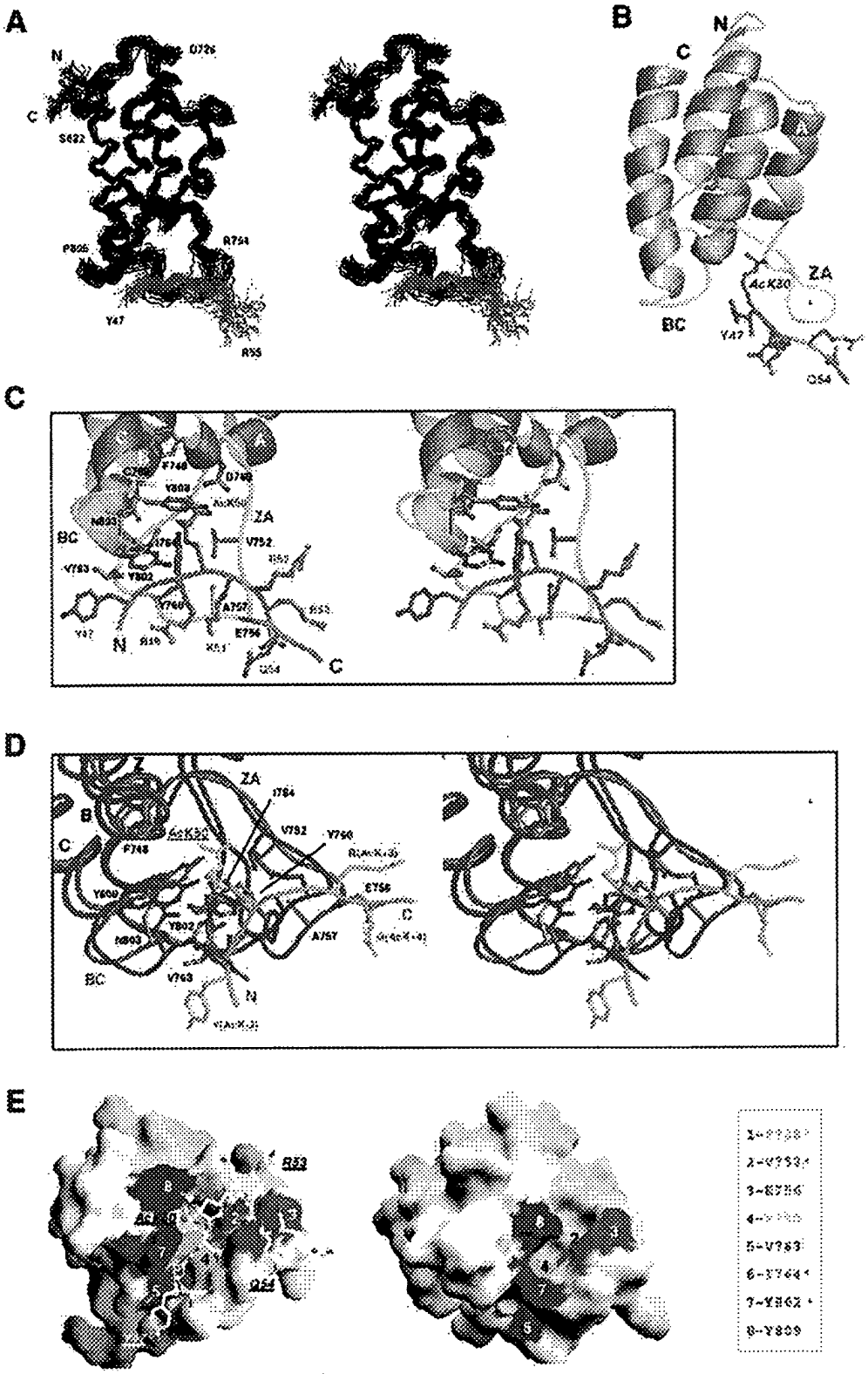


Figure 2. NMR Structure of the PCAF Bromodomain/Tat AcK50 Peptide Complex

(A) Stereoview of the backbone atoms (N, Cα, and C') of 25 superimposed NMR-derived structures of the PCAF bromodomain (black) (showing residues 719–830) in complex with the Tat AcK50 peptide (green) (showing residues 46–55). Note that amino acid residues in the Tat peptide are described either according to their relative positions with respect to the acetyl-lysine in the sequence or for clarity numbered by their specific positions in the protein sequence of Tat.

Table 1. Summary of NMR Structural Statistics

Total Experimental Restraints	2903	
Total NOE Distance Restraints[a]	2822	
Protein		
Total ambiguous	122	
Total unambiguous	2590	
Intraresidue	1093	
Interresidue		
Sequential ($\|i - j\| = 1$)	480	
Medium ($2 \leq \|i - j\| \leq 4$)	547	
Long range ($\|i - j\| > 4$)	470	
Peptide	32	
Intermolecular	78	
Hydrogen Bond Restraints	28	
Dihedral Angle Restraints	53	
Final Energies (kcal·mol^{-1})		
E_{Total}	366.4 ± 31.1	
E_{NOE}	58.0 ± 12.6	
$E_{Dihedral}$	0.6 ± 0.3	
E_{L-J}[b]	−569.5 ± 22.4	
	Protein/Peptide Complex[f]	Secondary Structure
Ramachandran Plot (%)		
Most favorable region	72.1 ± 2.3	92.0 ± 3.0
Additionally allowed region	22.9 ± 2.4	7.4 ± 3.1
Generously allowed region	3.6 ± 1.4	0.6 ± 0.1
Disallowed region	1.3 ± 0.6	0.0 ± 0.0
Cartesian coordinate RMSDs (Å)[c]		
Backbone atoms (N, Cα, and C′)[d]	0.66 ± 0.14	0.39 ± 0.05
Heavy atoms[d]	1.25 ± 0.18	0.96 ± 0.08
Backbone atoms (N, Cα, and C′)[e]	0.50 ± 0.16	
Heavy atoms[e]	1.83 ± 0.50	
Backbone atoms (N, Cα, and C′)[f]	0.72 ± 0.15	0.54 ± 0.09
Heavy atoms[f]	1.39 ± 0.20	1.25 ± 0.16

[a] Of the total 2822 NOE-derived distance restraints, 341 were obtained by using ARIA program, of which 122 are classified as ambiguous NOEs. The latter NOE signals in the NMR spectra match with more than one proton atom in both the chemical shift assignment and the final NMR structures.
[b] The Lennard-Jones potential was not used during any refinement stage.
[c] None of these final structures exhibit NOE-derived distance restraint violations greater than 0.3 Å or dihedral angle restraint violations greater than 5°.
[d] Protein residues 723–830.
[e] Peptide residues 47–52 and 53–54.
[f] Protein residues 723–830 and peptide residues 47–52 and 53–54.

residues (Figure 1B; see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/9/3/575/DC1). For instance, aromatic protons of Y802 in the BC loop and Y760 in the ZA loop show numerous long-range NOEs in the free form, which become completely absent in the peptide-bound form (see Supplemental Figures S2A, S2B, and S2C at http://www.molecule.org/cgi/content/full/9/3/575/DC1). These changes of NOE patterns are reflected in a ~90° rotational flip of the aromatic ring of Y802, which opens a channel lined by the ZA and BC loops to grasp the peptide through intermolecular interactions such as those observed between Y(AcK-3) and V763 (Figure 2D). Changes of loop conformation in the ZA and BC loops also result in exposing otherwise almost completely buried protein residues such as F748, V752, and I764 for direct peptide recognition (Figure 2E). Supporting NMR data include: (1) the methyl group (δ1) of I764 in the ZA loop shows a NOE cross peak to H^{α} of Y802, only in the free but not in the complex form; and (2) the methyl group of A757 in the ZA loop changes its spatial position from being close to the aromatic ε protons of Y802 (Y802.ε) in the free form to being proximal to Y761.ε upon binding to the Tat peptide (see Supplemental Figures S2D and S2E at

(B) Ribbons (Carson, 1991) representation of the average minimized NMR structure of the PCAF bromodomain/Tat peptide complex.
(C) Stereoview of the Tat binding site in the bromodomain showing side chains of the protein (green) and peptide (blue) residues that are directly involved intermolecular interactions.
(D) Stereoview of superimposition of the free (green) and ligand-bound (blue) structures of PCAF bromodomain showing side chain conformation of the residues in the Tat peptide binding site. The residues of the Tat peptide are colored in orange.
(E) Surface representation of the Tat binding site of the bromodomain in ligand-bound (left) and free form (right). Protein residues important in ligand recognition are colored with the same color scheme in both structures. Residues indicated by an asterisk are almost completely buried in the free form structure.

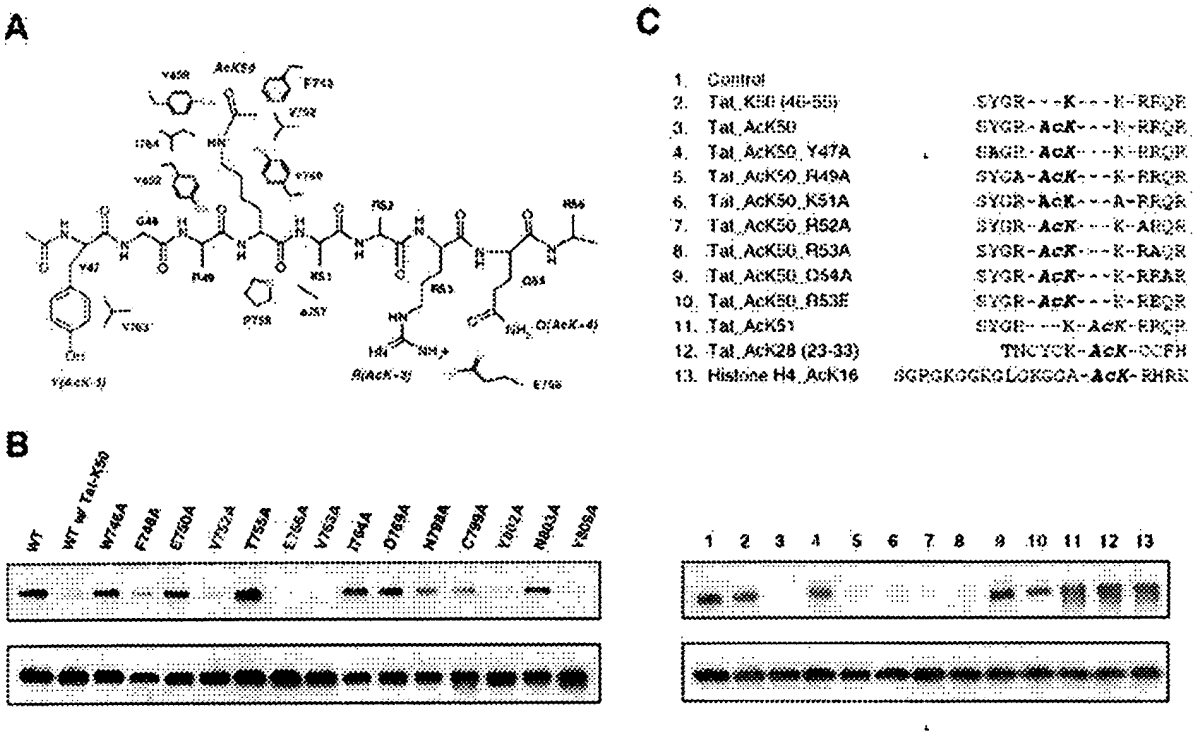


Figure 3. Mutational Analyses of PCAF Bromodomain Binding to HIV-1 Tat

(A) Schematic diagram showing amino acid residues involved in the protein/peptide interface.

(B) Effect of point mutation of protein residues on Tat AcK50 peptide binding. Western blot with anti-GST antibodies shows binding of the GST-fusion PCAF bromodomain proteins to the biotinylated Tat AcK50 peptide immobilized on streptavidin agarose beads (upper panel). The lower panel indicates a relatively equal amount of bromodomain proteins used in each binding experiment. Protein residues highlighted in red exhibited a significant reduction in binding to the Tat AcK50 peptide due to an alanine substitution.

(C) Mutational analysis of Tat peptide residues. Mutation effect was assessed by a peptide competition assay using anti-GST Western blot, in which a nonbiotinylated peptide competes with the biotinylated wild-type Tat AcK50 peptide for binding to the GST-fusion PCAF bromodomain. Numerals above the upper panel indicate a specific peptide used in the competition assay. The numerals in red refer to mutant Tat peptides that showed a significant reduction in binding to the bromodomain in the competition assay. The lower panel shows the relatively equal amount of bromodomain proteins used in each study. For clarity, the peptide residues are numbered according to their positions in the Tat protein sequence.

http://www.molecule.org/cgi/content/full/9/3/575/DC1). Together, our NMR data strongly suggest that conformational changes of protein residues in the ligand binding site are directly coupled with the highly selective interactions between PCAF bromodomain and acetylated Tat.

Specificity of PCAF Bromodomain and Tat Recognition

To determine the relative contributions of bromodomain residues in Tat binding site (Figure 3A), we examined its mutant proteins for binding to an N-terminal biotinylated Tat AcK50 peptide immobilized onto streptavidin agarose beads. Mutation of bromodomain residues W746, E750, T755, I764, D769, N798, C799, or N803 to alanine did not affect peptide binding, whereas proteins containing an alanine mutation at F748, V752, Y802, or Y809 showed a major reduction or nearly complete loss in Tat binding (Figure 3B). Moreover, alanine substitution of V763 or E756 almost completely abrogated peptide association, underlining the importance of their specific interactions with peptide residues Y(AcK−3) and Q(AcK+4). It is important to note that since both V763 and E756 are solvent exposed and located in loop regions of the structure (Figure 2D), their individual mutation to alanine unlikely causes major conformational disruption of the protein. Mutation results of these protein residues are consistent with the NMR structure of the complex and confirm their direct interactions with the acetyl-lysine and/or its flanking residues in the Tat peptide.

To further identify determinants in Tat sequence for PCAF bromodomain recognition, we synthesized mutant peptides and tested their binding to the protein in a peptide competition assay. Because of the high sensitivity of this detection method, the binding study was performed at a protein concentration (10 μM) much lower than that required for NMR study (~200 μM), ensuring specificity of protein-peptide interactions. As anticipated, lysine-acetylated peptides derived from Tat at K51 or K28 (lanes 11 and 12 in Figure 3C) or from histone H4 at K16 (lane 13) showed almost no competition against Tat AcK50 peptide in PCAF bromodomain binding, demonstrating that the latter interaction is of high affinity and specificity. Alanine substitution of resi-

dues R(AcK−1), K(AcK+1), R(AcK+2), or R(AcK+3) in Tat AcK50 peptide slightly weakened its binding to the bromodomain. Conversely, change of Y(AcK−3) (lane 4) or Q(AcK+4) (lane 9) to alanine caused a nearly complete loss of bromodomain binding, confirming the importance of their pairwise interactions with V763 and E756 for Tat-PCAF association. Finally, while mutation of R(AcK+3) to alanine (lane 8) did not significantly alter Tat binding to the bromodomain, its substitution to glutamic acid (lane 10) exhibited a marked reduction in the protein/peptide interaction. The effect of the latter mutation is likely due to an electrostatic repulsion between the glutamate and E756 of the protein. Together, these results explain the structural basis for the highly selective nature of PCAF and lysine-acetylated Tat association, which requires specific interactions of the bromodomain with AcK50 and its flanking residues, including Y(AcK−3), R(AcK+3), and Q(AcK+4).

PCAF Bromodomain Competing against TAR RNA for Binding to Lysine-Acetylated Tat

The arginine-rich motif containing R52 and R53 in Tat is also known to interact with the HIV-1 TAR RNA element (Aboul-ela et al., 1995; Long and Crothers, 1999; Rana and Jeang, 1999). Tat acetylation at K50 by p300/CBP promotes Tat dissociation from TAR RNA in early transcriptional elongation (Deng et al., 2000; Kieman et al., 1999). To determine whether lysine acetylation directly affects Tat association with TAR RNA, we performed an NMR study of a 27 nucleotide HIV-1 TAR RNA binding to Tat peptides containing either K50 or AcK50. Our results showed that TAR RNA bound to the nonacetylated Tat peptide with a subnanomolar affinity (K_D), in agreement with results reported previously (Aboul-ela et al., 1995; Long and Crothers, 1999), and that K50 acetylation of Tat resulted in a significant reduction of its affinity to TAR RNA (data not shown). More strikingly, we found that PCAF bromodomain competes effectively against TAR RNA for binding to Tat AcK50 peptide (Figures 4A and 4B), suggesting that the binding affinity (K_D) of the latter interaction is on the order of low micromolar. This observation may be explained by possible conformational change of the peptide residues due to acetylation at K50 or involvement of R53 of Tat in both interactions. These results strongly imply that the PCAF bromodomain interaction with AcK50 on Tat not only contributes to Tat-PCAF association but also to the release of lysine-acetylated Tat from TAR RNA association, leading to Tat-mediated HIV-1 transcriptional activation.

Differences of Ligand Selectivity of Bromodomains

Structural comparison of bromodomains from PCAF and other proteins extends our understanding of differences in ligand selectivity. Recent structures of bromodomains from human GCN5 (Hudson et al., 2000) and *Saccharomyces cerevisiae* GCN5p (Owen et al., 2000), and the double bromodomain module of human TAF_II250 (Jacobson et al., 2000), reinforce the notion that the left-handed four-helix bundle fold of the PCAF bromdomain is conserved in the bromodomain family (Dhalluin et al., 1999). Structural similarity is high for the four helices with pairwise root-mean-square deviations of 0.7–1.8 Å for the backbone Cα atoms. The majority of structural deviations are localized in the loop regions, particularly in the ZA and BC loops (see Supplemental Figure S3 at http://www.molecule.org/cgi/content/full/9/3/575/DC1).

The crystal structure of scGCN5p bromodomain solved in complex with an acetylated peptide derived from histone H4 at K16 (A-AcK-RHRKILRNSIQGI) reveals that the mechanism of acetyl-lysine recognition is highly conserved in bromodomains—it involves a nearly identical set of corresponding conserved residues in the PCAF and scGCN5p bromodomains (Figures 5A and 5B) (Owen et al., 2000). In addition to the acetyl-lysine, scGCN5p bromodomain has a limited number of contacts with two residues at (AcK+2) and (AcK+3) in the H4 peptide. Binding of H(AcK+2) to aromatic rings of Y406 and F367 in scGCN5p is reminiscent of PCAF bromodomain recognition of Tat Y(AcK−3) through interactions with Y802 and V763, which are equivalent to the two scGCN5p residues. Because of this similar mode of molecular interaction, the two aromatic residues in the Tat and H4 peptides, which are located in very different positions with respect to the acetyl-lysine, are bound in a nearly identical position in the corresponding bromodomain structures (Figure 5A). High conservation of these residues in bromodomains (Figure 5B) suggests that selection of an aromatic or hydrophobic residue neighboring the acetyl-lysine is possibly conserved for many members of the bromodomain family.

It is important to note that while the major binding determinant in scGCN5p bromodomain-H4 complex is the acetyl-lysine (Owen et al., 2000), the highly specific association of PCAF bromodomain and Tat peptide is dependent on its interactions not only with the acetyl-lysine and Y(AcK−3) but also with residues on the other side of the acetyl-lysine at (AcK+3) and (AcK+4) (Figures 3B and 3C). These differences in the extent of ligand interactions explain why the Tat AcK50 peptide competes effectively against a similar histone H4 AcK16 peptide for binding to the PCAF bromodomain (Figure 3C, lane 13). Moreover, these differences in ligand selectivity provide an explanation for the striking differences in location and orientation of the bound peptides in the two bromodomains—the backbones of the Tat and H4 peptides lie in the two corresponding structures nearly antiparallel to each other (Figure 5A). Binding of A757 and E756 in the ZA loop to R(AcK+3) and Q(AcK+4) of the Tat peptide, which are completely lacking in the scGCN5 bromodomain-H4 complex, further explains why the PCAF bromodomain undergoes more extensive conformational changes in the ligand site than those seen in the GCN5 bromodomains (see Supplemental Figure S3 at http://www.molecule.org/cgi/content/full/9/3/575/DC1). While the biological relevance of the scGCN5 and histone H4 AcK16 interaction remains to be determined, a growing body of evidence, including previous reports (Benkirane et al., 1998; Deng et al., 2000), our present study of NMR structure and in vitro mutagenesis, and results from in vivo functional studies of Tat-mediated HIV-1 transcriptional activation (Figure 1C and M.O. and E.V., unpublished data), strongly support the biological relevance and importance for the highly selective association of PCAF bromodomain and acetylated Tat.

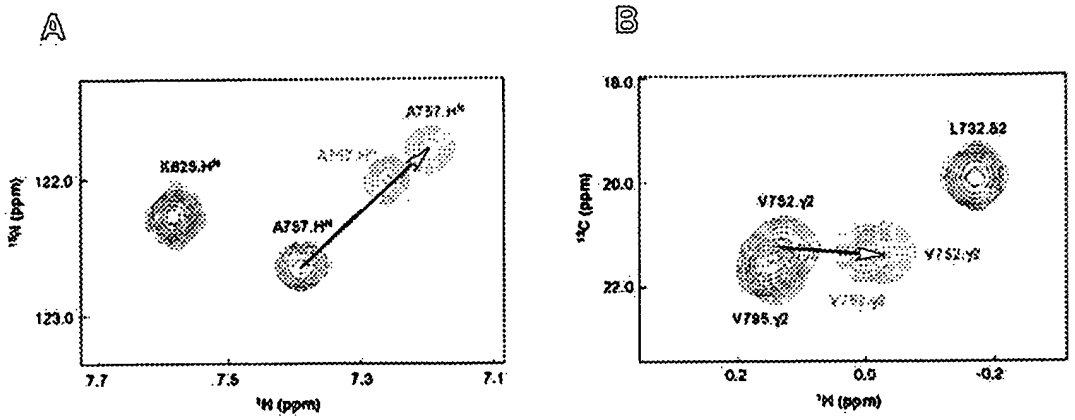


Figure 4. PCAF Bromodomain Competing against TAR RNA for Binding to Tat AcK50 Peptide

(A) Superimposition of a selected region of 2D ^{1}H-^{15}N HSQC spectra of a $^{13}C/^{15}N$-labeled PCAF bromodomain protein in the free form (black), in the presence of Tat AcK50 peptide (molar ratio of 1:1.2) (red), and in the presence of Tat AcK50 peptide and TAR RNA (molar ratio of 1:1.2:1 for protein:peptide:RNA) (blue). The spectra show chemical shift changes of the backbone amide resonances of protein residues due to peptide binding.

(B) Superimposition of 2D ^{1}H-^{13}C HSQC spectra of the PCAF bromodomain collected under the same conditions as described in (A). The NMR spectra exhibit chemical shift changes of the side chain methyl group resonances of protein residues due to peptide binding. The same color-coding scheme was used as in (A). Arrows indicate chemical shift changes of protein NMR resonances from the free form (black) to the Tat AcK50 peptide-bound form (red). Note that only the bromodomain residues (i.e., A757 and V752) that directly interact with the Tat peptide, as shown in the three-dimensional structure, exhibited major chemical shift changes upon peptide binding or in competing against TAR RNA for binding to the Tat peptide. More importantly, addition of TAR RNA causes only small shifts of the protein signals from the Tat peptide-bound position toward the free form position, suggesting that the PCAF bromodomain competes effectively against TAR RNA for binding to the lysine-acetylated Tat peptide. We observed by NMR no significant nonspecific interactions between the protein and TAR RNA under these conditions.

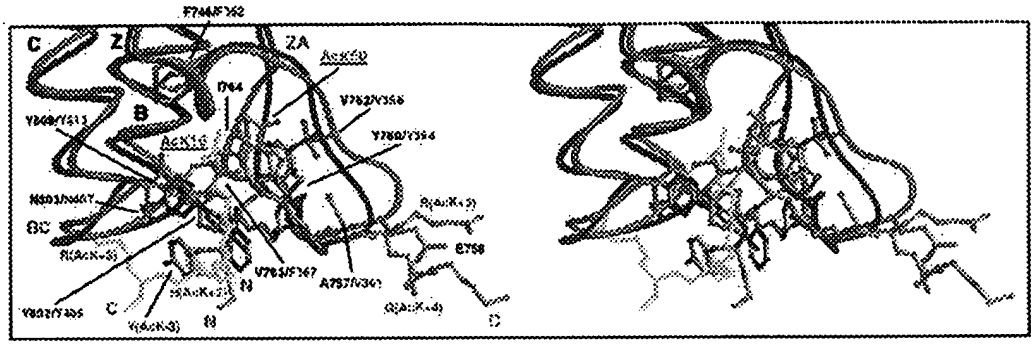
Since bromodomain residues important for acetyl-lysine recognition are largely conserved, binding of acetyl-lysine on a protein is likely a general biochemical function for bromodomains. However, differences in ligand selectivity may be attributed to a few but important differences in bromodomain sequences (Figure 5B), which include (1) variations in the ZA loop such as relatively low sequence conservation and amino acid deletion or insertion; and (2) variation of bromodomain residues that are involved in direct interactions with residues surrounding acetyl-lysine in a target protein. For instance, E756 in the bromodomain of PCAF is unique and only present in a small subset of bromodomains including GCN5. An analogous residue in the structurally similar bromodomain of CBP or p300 (Y.H. and M.-M.Z., unpublished data) is a leucine followed by a 2 amino acid insertion, which are present in a small subfamily of bromodomains (Figure 5B) (Jeanmougin et al., 1997). Moreover, a short helix corresponding to the AWPFM sequence in the ZA loop of PCAF (residues 745–749) is likely completely missing in TIF1β bromodomain due to amino acid deletion, and E756 of PCAF is substituted with a two residue AT motif in the sequence. Together, these findings explain why bromodomains from CBP and TIF1β did not interact with Tat AcK50 peptide (Figure 1A).

Human GCN5-S, a shorter version of hGCN5 that contains only the HAT domain and the bromodomain but lacks the N-terminal p300/CBP binding domain due to an alternative RNA splicing (Schiltz and Nakatani, 2000; Yamauchi et al., 2000), has been recently reported to interact with HIV Tat in vitro (Col et al., 2001). This Tat interaction involves both the HAT domain and the bromodomain of hGCN5-S. While the specific binding site on Tat by the hGCN5 bromodomain and the question of whether this bromodomain interaction is dependent on Tat lysine acetylation remain to be determined, our data reported here suggest that the hGCN5 bromodomain may possess ligand selectivity similar to that of the structurally homologous bromodomain of PCAF.

Conclusion

Tat-stimulated transcriptional activation of integrated HIV-1 genomes defines the rate-limiting step for viral replication (Adams et al., 1994; Benkirane et al., 1998). Tat synergy with histone acetyltransferases and its recruitment of PCAF via a bromodomain interaction, as we described in this study, support the notion that Tat transactivation of HIV-1 chromosomal transcription proceeds via chromatin remodeling (Deng et al., 2000; Kiernan et al., 1999; Ott et al., 1999). Our findings may explain why deletion of the PCAF C-terminal region comprising the bromodomain potently abrogated Tat transactivation of integrated but not unintegrated HIV-1 LTR (Benkirane et al., 1998). Our study reinforces the concept that bromodomain and acetyl-lysine recognition could serve as a pivotal mechanism for controlling protein-protein interactions in chromatin remodeling as well as other cellular processes including viral life cycle (Dyson et al., 2001; Winston and Allis, 1999), and that differences

A



B

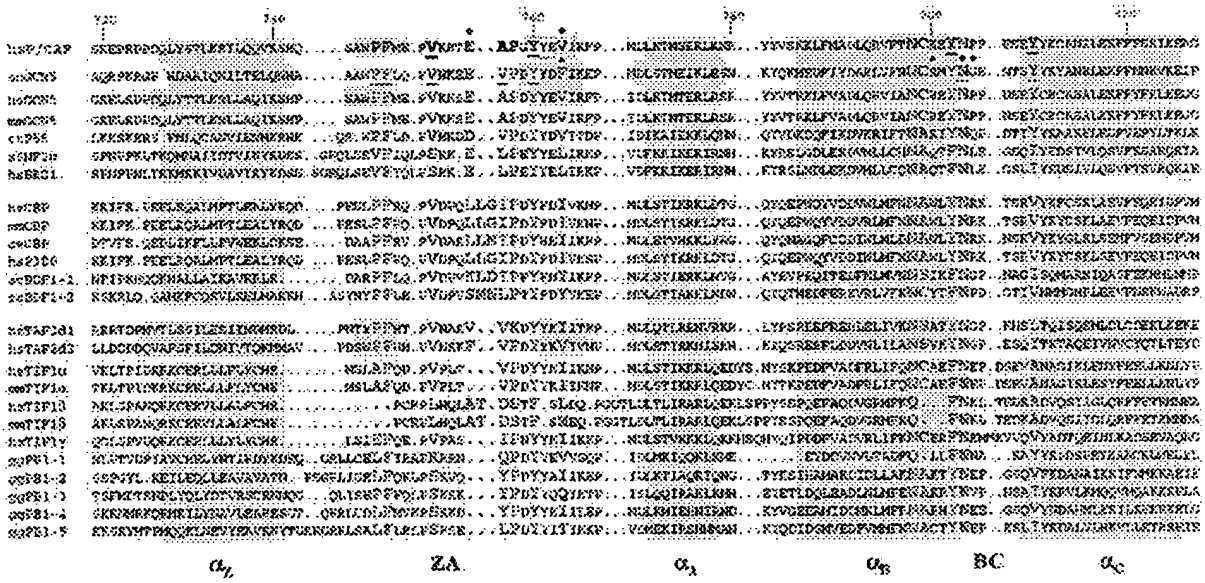


Figure 5. Differences in Ligand Selectivity of Bromodomains

(A) Stereoview of superimposition of the structures of the PCAF bromodomain-Tat peptide complex (blue) and the scGCN5 bromodomain-H4 peptide complex (red) showing conformational differences of protein residues in the peptide binding sites. The lysine-acetylated peptides of HIV-1 Tat and histone H4 are shown in green and orange, respectively. The residues of the PCAF and scGCN5 bromodomains are numbered according to protein sequences and color-coded in black and red, respectively. The corresponding conserved residues in the two bromodomains are annotated together. The amino acid residues of the Tat and H4 peptides are described according to their position with respect to the acetyl-lysine in the corresponding peptide sequences. The structures were superimposed on the four helices of the two bromodomains in Insight and the figure was prepared with Ribbons.

(B) Sequence alignment of a selected number of bromodomains. The sequences were aligned based on the experimentally determined three-dimensional structures of five bromodomains, highlighted in yellow. Note that in the PCAF bromodomain, a short helix in the ZA loop comprising the YYEVI sequence (residues 760–764) (boxed by dashed lines) was only observed in the free form but not in the Tat peptide-bound form. The predicted secondary structures in the corresponding regions of other bromodomains are shown in green. Because of relatively high variations in amino acid sequence in the ZA loop, prediction of secondary structures was omitted. Bromodomains are grouped on the basis of the predicted sequence and/or structural similarities. Residue numbers of the PCAF bromodomain are indicated above its sequence. Three absolutely conserved residues, corresponding to P751, P767, and N803 in the PCAF bromodomain, are shown in purple. Highly conserved residues are colored in blue. The residues in the PCAF bromodomain that directly interact with the Tat peptide, as determined by intermolecular NOEs, are displayed in a larger font size. The residues essential for the acetyl-lysine binding are underlined, and the residues important for ligand selectivity through interactions with the peptide residues flanking the acetyl-lysine are highlighted by red asterisks. The protein residues contacting the acetyl-lysine in the scGCN5 bromodomain-H4 peptide complex are underlined, and residues contacting other parts of the peptide are indicated by red dots.

in ligand selectivity of conserved protein modular domains can be achieved by evolutionary changes of amino acid sequences in the ligand binding site. The new knowledge of the structural basis of PCAF bromodomain and Tat recognition should aid in the design of small molecules that can be used to block this specific

interaction in order to disrupt HIV-1 transcriptional activation and replication.

Experimental Procedures

Sample Preparation

The PCAF bromodomain (residues 719–832) was expressed in *Escherichia coli* BL21(DE3) cells using the pET14b vector (Novagen) (Dhalluin et al., 1999). Isotope-labeled bromodomain proteins were prepared from cells grown on a minimal medium containing $^{15}NH_4Cl$ with or without $^{13}C_6$-glucose in either H_2O or 75% ^2H_2O. The protein was purified by affinity chromatography on a nickel-IDA column (Invitrogen), followed by the removal of poly-His tag by thrombin cleavage. GST-fusion bromodomains from PCAF, CBP, and TIF1β were expressed in *E. coli* BL21 (DE3) codon plus cells using the pGEX4T-3 vector (Pharmacia) and purified with a glutathione sepharose column. NMR spectra of the recombinant CBP and TIF1β proteins were acquired to ensure that they were properly folded and functional (see Supplemental Figure S4 at http://www.molecule.org/cgi/content/full/9/3/575/DC1). The acetyl-lysine-containing peptides were prepared on a MilliGen 9050 peptide synthesizer (Perkin Elmer) using Fmoc/HBTU chemistry. Acetyl-lysine was incorporated using the reagent Fmoc-Ac-Lys with HBTU/DIPEA activation. The HIV-1 TAR RNA was obtained from Dharmacon Research, Inc. (Lafayette, CO).

NMR Spectroscopy

NMR samples contained a protein/peptide complex of 0.5 mM in 100 mM phosphate buffer (pH 6.5) containing 5 mM perdeuterated DTT and 0.5 mM EDTA in $H_2O/^2H_2O$ (9/1) or ^2H_2O. All NMR spectra were acquired at 30°C on a Bruker 500 or 600 MHz NMR spectrometer. The ^1H, ^{13}C, and ^{15}N resonances of the protein backbone and side chain atoms were assigned by using a standard set of triple-resonance experiments (Sattler et al., 1999) with a uniformly $^{13}C/^{15}N$-labeled and 75% deuterated protein in complex with an unlabeled peptide. The distance restraints were obtained from ^{13}C- or ^{15}N-edited three-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) spectra (Clore and Gronenborn, 1994). ϕ-angle restraints were determined based on the $^3J_{HN,H\alpha}$ coupling constants measured in a 3D HNHA spectrum (Clore and Gronenborn, 1994). Slowly exchanging amide protons were identified from a series of 2D ^{15}N-HSQC spectra recorded after the H_2O buffer was changed to a ^2H_2O buffer, which were used together with the initial structures calculated with only NOE-derived distance restraints to generate hydrogen-bond distance restraints in final structure calculations. The intermolecular NOEs were detected in ^{13}C-edited (F_1), $^{13}C/^{15}N$-filtered (F_3) 3D NOESY spectrum (Clore and Gronenborn, 1994). All NMR spectra were processed with the NMRPipe program (Delaglio et al., 1995) and analyzed using NMRView (Johnson and Blevins, 1994). NMR binding studies of Tat peptides and TAR RNA interactions were performed in the phosphate buffer (pH 6.5) containing 200 mM NaCl to minimize any nonspecific interactions.

Structure Calculations

Structures of the protein/peptide complex were calculated with a distance geometry-simulated annealing protocol using the X-PLOR program (Brunger, 1993). A total of 2359 manually assigned NOE-derived distance restraints were used in initial structure calculations. The ARIA (Nilges and O'Donoghue, 1998)-assigned distance restraints agree with the structures calculated using only the manually determined NOE distance restraints, 28 hydrogen-bond distance restraints, and 53 ϕ angle restraints. The final structure calculations employed a total of 2903 NMR experimental restraints from the manual and the ARIA-assisted assignments, including 2700 unambiguous intramolecular and 78 intermolecular NOE distance restraints. The distance restraint force constant was 50 kcal mol^{-1} $Å^{-2}$, and no NOE was violated by more than 0.3 Å. The torsion restraint force constant was 200 kcal mol^{-1} rad^{-2}, and no dihedral angle restraint was violated by more than 5°. While only the covalent geometry terms, NOE, torsion, and repulsive van der Waals terms were used in the structure refinement, a large, negative Lennard-Jones potential energy was observed (−569.5 ± 22.4 kcal mol^{-1}), indicating good nonbonded geometry of the structure. Procheck (Laskowski et al., 1996) analysis indicated that over 98% of the protein and peptide residues are in allowed regions of the Ramachandran map.

Mutational Analysis

Site-directed mutant proteins of PCAF bromodomain were prepared with the QuickChange kit (Stratagene). DNA sequencing confirmed the desired mutations. The GST-fusion bromodomains (10 μM) of PCAF, CBP, or TIF1β were incubated with an N-terminal biotinylated and lysine-acetylated Tat peptide (50 μM) in 50 mM Tris buffer (pH 7.5), containing 50 mM NaCl, 0.1% BSA, and 1 mM DTT at 22°C for 2 hr. Streptavidin agarose (10 μL) was added to the mixture, and the beads were washed in the Tris buffer containing 500 mM NaCl and 0.1% NP-40. Proteins eluted from the agarose beads were separated by SDS-PAGE and visualized by Western blotting using anti-GST antibody (Sigma) and horseradish-peroxidase-conjugated goat anti-rabbit IgG. Peptide competition assay was performed by incubating a nonbiotinylated peptide with the PCAF bromodomain and the biotinylated Tat AcK50 peptide. The molar ratio of the former and latter peptides in the mixture was kept at 1:2.

Plasmid Constructs

The mammalian expression vectors for the PCAF and CBP bromodomains were constructed as follows. Coding sequence for the PCAF bromodomain (residues 719–832) was subcloned into EcoRI-XhoI sites of pCMV-HA vector (Clontech). The CBP bromodomain (residues 1082–1197) was subcloned into BamHI-XhoI sites of pCMV-FLAG vector (Stratagene). The expression vectors for the full-length PCAF (pCI-FLAG-PCAF) (Li et al., 2000), the full-length CBP (pRSV-HA-CBP) (Kwok et al., 1996), HIV-1 Tat (pcTat), and the HIV-1 LTR-luciferase reporter construct (pHIV-LTR-Luc) (Bieniasz et al., 1998; Madore and Cullen, 1993) have been previously described.

Cell Culture and Transfections

Human 293T cells were propagated in Dulbecco's modified Eagle's medium with 10% fetal calf serum and transfected using the calcium phosphate coprecipitation method. Amounts of plasmid DNA used in cell transfections are as described in the legend to Figure 1C. The transfected 293T cells were lysed 24 hr after transfection and assayed for luciferase activity of the cell extracts using a luciferase-based assay system (Promega) (Bieniasz et al., 1998; Madore and Cullen, 1993). Luciferase activities derived from HIV-1 LTR were normalized to a cotransfected vector expressing β-galactosidase. The expression level of the transfected proteins was examined by Western blotting using monoclonal antibodies to HA (Roche Diagnostics), FLAG (Stratagene), or β-actin (Sigma), and rabbit polyclonal antibodies to the PCAF bromodomain or the CBP bromodomain (see Supplemental Figure S5 at http://www.molecule.org/cgi/content/full/9/3/575/DC1).

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Accession Numbers

Coordinates for the NMR three-dimensional structure of the PCAF bromodomain/HIV-1 Tat peptide complex have been deposited in the Brookhaven Protein Data Bank under the accession code 1JM4.